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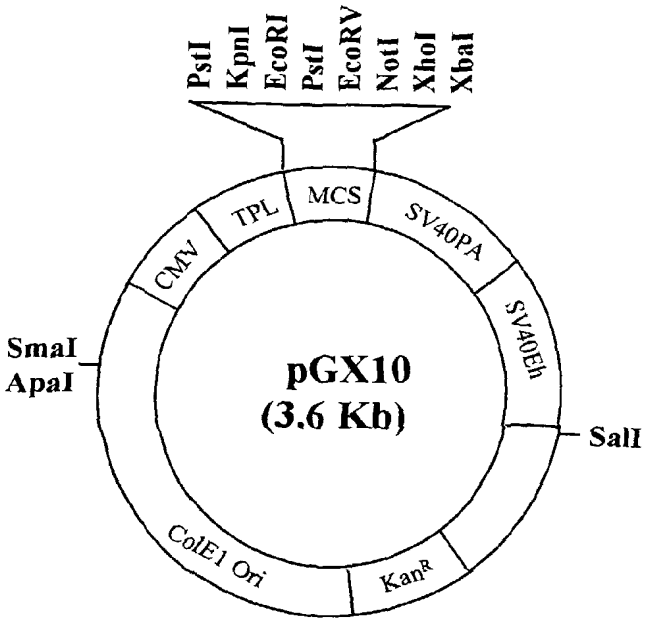
(54) Title: SIVMAC239 IMMUNOGENIC PLASMIDS AND AIDS DNA VACCINE CONTAINING THE SAME



(57) Abstract: The present invention relates to immunogenic plasmids showing excellent expression efficiency of immunogens and immune efficacy in the SIVmac239/rhesus monkey model and AIDS human patients. Also, the present invention relates to DNA vaccines for prophylaxis or treatment of AIDS containing the above immunogenic plasmids.

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

SIVmac239 IMMUNOGENIC PLASMIDS AND AIDS DNA VACCINE CONTAINING THE SAME

Technical Field

The present invention relates to an AIDS DNA vaccine. More particularly, the present invention relates to SIVmac239 and HIV immunogenic plasmids and AIDS DNA vaccines containing the plasmids.

Background Art

DNA vaccination is the most recently developed vaccination method. Ertl et al. disclosed a method for administering a live or dead vaccine in a strategy for common immunization (Ertl et al., J. Immunol. 156, 3579-3582 (1996)). Hassett et al. warned that a live vaccine may be dangerous in case of a human or animal patient with lowered immunity and or pregnant patient, since the antigen in the vaccine can revert to or mutate into a pathogenic form, though it can generally elicit an effective immune response in a vaccinated body (Hassett et al., Trends in Microbiol. 8, 307-312 (1996)). Recently, Tang et al. found that an expression plasmid encoding an antigenic protein that is directly injected into mouse can induce an antibody response (Tang, D.C., et al., Nature (Lond.) 356, 152-154 (1992)). This suggested that injection of naked DNA may express an antigen type capable of inducing an immune response. Also, there has been a report that intramuscular injection of plasmid DNA encoding nucleoprotein of influenza may protect a mouse infected with a live influenza virus, thereby opening a

new era for development of vaccines (Ulmer, J.B., et al., Science 259, 1745-1749 (1993); and Fynan, E.F., et al., Proc. Nat. Acad. Sci. USA 90, 11, 478-482 (1993)). Also, it has been shown that immunization with plasmid DNA may activate both humoral immunity and cellular immunity, including production of antigen-specific CD8+ cytotoxic T cells as well as CD4+ T helper cell (Donnellym, J.J., et al., Ann Rev. Immunol. 15, 617-648 (1997)). In addition, Felgner et al. disclosed a method for delivering an isolated polynucleotide such as DNA or RNA, in which the polynucleotide is intramuscularly administered to mammals so that muscle cells absorb the polynucleotide, the method having therapeutic effects in the mammals (US PAT. No.5,589,466).

In general, it is thought that the protection level achieved by a DNA vaccine is lower than those observed in cases naturally recovered from infection with virus (Manickan et al., Critical Review Immunol. 17, 139-154 (1997)) but is similar to levels induced by conventional protein-specific antigen vaccines and dead or attenuated virus vaccines. However, a DNA vaccine comprising naked plasmid DNA has some merits, as compared to immunization methods depending upon injection of purified or recombinant protein, or attenuated live or recombinant virus, as follows. (1) Genes encoding a particular tumor antigen and/or immunomodulatory cytokine can be introduced at the same time, since one or more genes can be easily introduced at the same time. (2) Only a desired gene can be transcribed without immunological interference from virus protein *in vivo* and *in vitro*. (3) There is no risk of recombination, which can occur when using replication-defective viral vectors. (4) It is substantially impossible for foreign DNA to be inserted into the host genome due to the transient nature of gene transfer. (5) DNA vectors which will be used in this

method can be readily prepared. (6) It is possible to simultaneously induce humoral and cellular immune responses against a variety of antigens and to control characteristics of immune responses by simultaneously delivering genes encoding immunoregulatory cytokines and costimulatory molecules.

AIDS (Acquired Immune Deficiency Syndrom) is a disease which has been continuously studied since the first diagnosis in 1981. Upon infection with HIV, the number of CD4+ T cells is dramatically reduced, causing serious damage to the immune system. As a result, complex opportunistic infections or neoplasias arise due to the compromised immune system. So far, in order to treat AIDS, numerous types of vaccines has been developed, including for example, inactivated whole virus vaccines, live recombinant virus vaccines, attenuated virus vaccines, specific antigen subunit vaccines, synthetic peptide vaccines and anti-idiotype antibodies, etc. For example, an inactivated whole virus vaccine is disclosed in US PAT. No. 5,698,432 to John Sidney, an attenuated virus vaccine is disclosed in US PAT. No. 6,004,799 to Luciw et al., specific antigen subunit vaccines are disclosed in US PAT. No. 6,331,404 to Bermann et al., US PAT No. 6,083,504 to Cotropia, and a report by Dolin et al. (Ann Intern. Med., 114, 119-127 (1991)), synthetic peptide vaccines are disclosed in US PAT. No. 6,139,843 to Rubinstein et al. and US PAT. No. 5,817,318 to Sia et al. However, the methods using these vaccines have problems, for example, evasion of immune response *in vivo*, such as HIV escaping immune recognition through its mutation, and risk of infection due to recovery of pathogenicity of a virus vector which has been administered as an attenuated vaccine. Furthermore, the methods failed to show desired prophylactic or therapeutic effects versus AIDS.

To the contrary, it has been found that plasmid DNA, when administered to

primates, induced humoral immune response and cell-mediated immune respone, and effectively induced Th1 (T helper-1) bias immune response and CTL response, which are known to be important for protection against viruses such as HIV-1, especially in small animals, monkeys, chimpanzees and humans. There are many DNA vaccines against AIDS. For example, EP 0276591 disclosed a vaccine consisting of a viral vector and recombinant DNA coding for the p25 protein of the AIDS virus. The viral vector is characterized by comprising a part of the genome of a vector virus; the complete gag gene or one of its fragments, especially a gene coding for the p25 protein or a gene coding for the p18 protein of the HIV virus responsible for AIDS; and elements ensuring the expression of these proteins in cells during culturing.

EP 0572737 disclosed a substantially pure HIV antigen comprising a Gag-Env fusion protein consisting of a Gag polypeptide fused at its C-end to an Env peptide.

FR 2596771 disclosed a viral vector characterized by comprising: a portion of the genome of a virus, a gene encoding one of the glycoproteins (gp) of the envelope of the virus responsible for AIDS; and elements ensuring the expression of this glycoprotein in cells.

WO 99277958 disclosed an AIDS vaccine based on HIV-1 Tat as immunogen, in which HIV-1 Tat is inoculated either as DNA and/or recombinant protein or as peptides; alone or in combination with other genes or viral gene products (Nef, Rev, Gag) or parts thereof; or in combination with various immunomodulant cytokines (IL-12, IL-15) or with the gene coding for an immunomodulant cytokine or part thereof. According to this patent, Tat, Nef, Rev, Gag and the immunomodulant cytokines are administered both as a mixture of recombinant proteins, peptides or fusion proteins (Tat/Nef, Tat/Rev, Tat/Gag, Tat/IL-12, Tat/IL-15), or as plasmid DNA.

Also, Roger Miller and Nava Sarver reported that when a Rhesus macaques monkey was inoculated with vif gene-deficient SIVmac239 wherein and the deficient virus was attenuated expression type, the monkeys formed a low level of antibody against SIVmac239 (Roger Miller and Nava Sarver, HIV Vif as a Therapeutic Target, DAIDS, NIAID, Sept. 18, 2000). However, it was described that the monkeys immunized with vif-deficient SIV cannot be protected against infection of wild-type SIV. H. Zhang, et al. disclosed that a mutated Gag could induce increased cytotoxicity in cells, as compared to a wild-type Gag (H. Zhang and L. Qiao, 184 HIV Gag DNA Vaccine, AIDS VACCINE, Foundation for AIDS Vaccine Research and Development, (2001)). However, such result was obtained in immunized mice with a DNA vaccine coding the mutated Gag.

As described in AIDS WEEKLY Plus, Monday, (AW) Conference Coverage (Retrovirus), March 24, 1997, Britta Wahren et al. showed that a plasmid containing attenuated HIV-1 nef, rev and tat genes, a gene encoding p24 structural protein and a gene encoding gp160 envelope precursor glycoproteins can induce cellular and humoral anti-HIV immune response. However, this study was carried using mice as an experimental animal.

It was also described in AIDS WEEKLY Plus, Monday, (AW) Conference Coverage (NCVDG), June 23, 1997 that Velpandi Ayyavoo and his colleagues showed that a naked DNA vaccine containing the attenuated HIV-1 genes, vif, nef, vpr and vpu could induce cellular and humoral anti-HIV immune response.

Amara et al. reported a study in which primates were immunized with a DNA vaccine containing many HIV and SIV genes (HIV-1 envelope, tat and rev genes and SIV gag, pol, vif, vpx and vpr genes) and a MVA (modified Vaccinia Ankara) booster

vaccine containing HIV- and SIV-derived genes (Amara R.R., et al., Science 292, 69-74, (2001). According to their report, it was demonstrated that the strong vaccine-induced immune response in rhesus macaques monkey/SHIV model can control viral replication and diseases progression.

However, in spite of various studies in the prior arts, there has been no descriptions or suggestions of a DNA vaccine capable of successfully preventing or treating AIDS. Therefore, there are still demands for a novel immunogenic plasmid with improved expression efficiency and immunogenicity, and an effective and safe DNA vaccine.

Disclosure of the Invention

Now, the inventors have developed immunogenic plasmids showing excellent expression efficiency and immunogenicity in SIVmac239/rhesus macaques monkey and AIDS human patients and DNA vaccines for prophylaxis or therapy of AIDS containing the same. We have developed two types of vaccine plasmids for AIDS in the previous study (Korean Patent Application Laid-Open No. 2001-0054338 and its corresponding US Patent Publication No. 2001004531). The first immunogenic plasmid comprises the vector pTV2 which was developed as a basic vector for a DNA vaccine, and the gag, dpol (corresponding to protease) and env genes derived from the virus SIVmac239 and the regulatory gene rev derived from the virus SIVmac239, but does not comprise the regulatory genes tat and nef; and the second immunogenic plasmid comprises the vector pTV2 which was developed as a basic vector for a DNA vaccine, and the SIVmac239 pol gene encoding reverse transcriptase (RT) and

integrase and a DNA sequence encoding a signal peptide of glycoprotein D (gD) of HSV (Herpes Simplex Virus) fused to the pol gene. These first and second immunogenic plasmids were developed to make up for defects of conventionally developed AIDS DNA vaccines in which the regulatory genes nef and tat, known to inhibit or disturb immune response *ex vivo*, were used, and the pol gene including many CTL epitopes which are known to be important for immunogenicity was not effectively used. According to the results of experiments, these immunogenic plasmids were shown to have excellent expression efficiencies and immune efficacies to some degrees in the SIVmac239/rhesus monkey model.

However, the inventors have now developed a novel ADIS DNA vaccine showing improved effects by augmenting our previous invention. One feature of the present invention is the vector pGX10 developed as a basic vector for a DNA vaccine. The pGX10 is confirmed to have a higher level of expression upon cell infection *ex vivo*, and to induce more excellent immune response in vivo (upon immunization of mouse), as compared to the vector pTV2. Also, in the present invention, in order to minimize the down-regulation of immune responses by the regulatory genes nef and tat, which are excluded in the previous invention and increase the expression efficiency of a gene to be introduced, portions of the nef and tat genes are used, not their full-length sequence, and the vector is designed so that the nef and tat genes are expressed through fusion with another regulatory gene, thereby achieving codon optimization. As a result, we have succeeded in developing immunogenic plasmids showing significantly increased expression efficiency of immunogens and immune efficacy as compared to the previous invention, and effective and safe DNA vaccines containing the same.

In accordance with one aspect, the present invention provides the vector

pGX10 as a basic vector for producing immunogenic plasmids which are used in the AIDS DNA vaccine according to the present invention.

In accordance with another aspect, the present invention provides the immunogenic plasmid pGX10-SIV/GE, which is used in the AIDS DNA vaccine composition according to the present invention, characterized by comprising the SIVmac239 gag, dpol and env genes and rev regulatory gene.

In accordance with another aspect, the present invention provides an immunogenic plasmid, which is used in the AIDS DNA vaccine composition according to the present invention and characterized by comprising the vector pGX10 and the SIVmac239 pol gene encoding reverse transcriptase (RT) and integrase (INT) and a DNA sequence encoding a signal peptide of secretory protein fused to the 3' end of the SIVmac239 pol gene which are operably linked to the vector.

In accordance with another aspect, the present invention provides an immunogenic plasmid, which is used in the AIDS DNA vaccine according to the present invention and characterized by comprising the SIVmac239 vif gene and the DNA sequence encoding a signal peptide of secretory protein and the SIVmac239 nef gene fused to the 3' and 5' ends of the SIVmac239 vif gene, respectively.

In accordance with another aspect, the present invention provides an immunogenic plasmid, which is used in the AIDS DNA vaccine according to the present invention and characterized by comprising any one of genes having from exon 1 to the full length of the SIVmac239 tat gene, and a signal sequence of secretory protein and the SIVmac239 vpx gene fused to the 3' and 5' ends of the SIVmac239 tat gene, respectively.

In accordance with another aspect, the present invention provides an

immunogenic plasmid, which is used in the AIDS DNA vaccine according to the present invention and characterized by comprising (i) the SIVmac239 vif gene and the DNA sequence encoding a signal peptide of secretory protein and the SIVmac239 nef gene fused to the 3' and 5' ends of the SIVmac239 vif gene, respectively, and (ii) any one of genes having from exon 1 to a full length of the SIVmac239 tat gene and the DNA sequence encoding a signal peptide of secretory protein and the SIVmac239 vpx gene fused to the 3' and 5' ends of the SIVmac239 tat gene, respectively.

In accordance with another aspect, the present invention provides the immunogenic plasmid pGX10-HIV/GE, which is used in the AIDS DNA vaccine (composition) according to the present invention, characterized by comprising the HIV-1 gag, protease and env genes and rev regulatory gene.

In accordance with another aspect, the present invention provides an immunogenic plasmid, which is used in the AIDS DNA vaccine (composition) according to the present invention and characterized by comprising the vector pGX10 and the HIV-1 pol gene encoding reverse transcriptase (RT) and integrase (INT) and the DNA sequence encoding a signal peptide of secretory protein fused to the 3' end of the HIV-1 pol gene which are operably linked to the vector.

In accordance with another aspect, the present invention provides an immunogenic plasmid, which is used in the AIDS DNA vaccine according to the present invention and characterized by comprising the HIV-1 vif gene and the DNA sequence encoding a signal peptide of secretory protein and the HIV-1 nef gene fused to the 3' and 5' ends of the HIV-1 vif gene, respectively.

In accordance with another aspect, the present invention provides an immunogenic plasmid, which is used in the AIDS DNA vaccine according to the

present invention and characterized by comprising any one of genes having from exon 1 to the full length of the HIV-1 tat gene, and the DNA sequence encoding a signal peptide of secretory protein and the HIV-1 vpx gene fused to the 3' and 5' ends of the HIV-1 tat gene, respectively.

In accordance with another aspect, the present invention provides an immunogenic plasmid, which is used in the AIDS DNA vaccine according to the present invention and characterized by comprising (i) the HIV-1 vif gene and the DNA sequence encoding a signal peptide of secretory protein and the HIV-1 nef gene fused to the 3' and 5' ends of the HIV-1 vif gene, respectively, and (ii) any one of genes having from exon 1 to a full length of the HIV-1 tat gene and the DNA sequence encoding a signal peptide of secretory protein and the HIV-1 vpx gene fused to the 3' and 5' ends of the HIV-1 tat gene, respectively.

In accordance with another aspect, the present invention provides the adjuvant plasmid pGX10-hIL-12m, which can be used in the AIDS DNA vaccine according to the present invention.

In accordance with another aspect, the present invention provides a DNA vaccine composition for prophylaxis or treatment of AIDS, characterized by comprising (i) plasmid pGX10-SIV/GE and (ii) plasmid comprising the vector pGX10 and the SIVmac239 pol gene encoding reverse transcriptase and integrase and the DNA sequence encoding a signal peptide of secretory protein fused to the 3' end of the SIVmac239 pol gene which are operably linked to the vector pGX10.

In accordance with another aspect, the present invention provides a DNA vaccine composition for prophylaxis or treatment of AIDS, characterized by comprising (i) plasmid comprising the SIVmac239 gag, dpol, env and rev genes, (ii)

plasmid comprising the SIVmac239 pol gene encoding reverse transcriptase and integrase and the DNA sequence encoding a signal peptide of secretory protein fused to the 3' end of the SIVmac239 pol gene, (iii) plasmid comprising the SIVmac239 vif gene and the DNA sequence encoding a signal peptide of secretory protein and the SIVmac239 nef gene fused to the 3' and 5' ends of the vif gene, respectively, and/or (iv) plasmid comprising any one of genes having from exon 1 to a full length of the SIVmac239 tat gene and the DNA sequence encoding a signal peptide of secretory protein and the SIVmac239 vpx gene fused to the 3' and 5' ends of the SIVmac239 tat gene, respectively.

In accordance with another aspect, the present invention provides a DNA vaccine composition for prophylaxis or treatment of AIDS, characterized by comprising (i) plasmid comprising the SIVmac239 gag, dpol, env and rev genes, (ii) plasmid comprising the SIVmac239 pol gene encoding reverse transcriptase and integrase and the DNA sequence encoding a signal peptide of secretory protein fused to the 3' end of the SIVmac239 pol gene, and (iii) plasmid comprising (a) SIVmac239 vif gene and the DNA sequence encoding a signal peptide of secretory protein and the SIVmac239 nef gene fused to the 3' and 5' ends of the SIVmac239 vif gene, respectively, and (b) any one of genes having from exon 1 to a full length of the SIVmac239 tat gene and the DNA sequence encoding a signal peptide of secretory protein and the SIVmac239 vpx gene fused to the 5' end of the SIVmac239 tat gene, respectively.

In accordance with another aspect, the present invention provides a DNA vaccine composition for prophylaxis or treatment of AIDS, characterized by comprising (i) plasmid pGX10-HIV/GE and (ii) plasmid comprising the vector pGX10

and the HIV-1 pol gene encoding reverse transcriptase and integrase and the DNA sequence encoding a signal peptide of secretory protein fused to the 3' end of the HIV-1 pol gene which are operably linked to the vector pGX10.

In accordance with another aspect, the present invention provides a DNA vaccine composition for prophylaxis or treatment of AIDS, characterized by comprising (i) plasmid comprising the HIV-1 gag, dpol, env and rev genes, (ii) plasmid comprising the HIV-1 pol gene encoding reverse transcriptase and integrase and the DNA sequence encoding a signal peptide of secretory protein fused to the 3' end of the HIV-1 pol gene, (iii) plasmid comprising the HIV-1 vif gene and the DNA sequence encoding a signal peptide of secretory protein and the HIV-1 nef gene fused to the 3' and 5' ends of the vif gene, respectively, and/or (iv) plasmid comprising any one of genes having from exon 1 to a full length of the HIV-1 tat gene and the DNA sequence encoding a signal peptide of secretory protein and the HIV-1 vpx gene fused to the 3' and 5' ends of the HIV-1 tat gene, respectively.

In accordance with another aspect, the present invention provides a DNA vaccine composition for prophylaxis or treatment of AIDS, characterized by comprising (i) plasmid comprising the HIV-1 gag, dpol, env and rev genes, (ii) plasmid comprising the HIV-1 pol gene encoding reverse transcriptase and integrase and the DNA sequence encoding a signal peptide of secretory protein fused to the 3' end of the HIV-1 pol gene, and (iii) plasmid comprising (a) HIV-1 vif gene and the DNA sequence encoding a signal peptide of secretory protein and the HIV-1 nef gene fused to the 3' and 5' ends of the HIV-1 vif gene, respectively, and (b) any one of genes having from exon 1 to a full length of the HIV-1 tat gene and the DNA sequence encoding a signal peptide of secretory protein and the HIV-1 vpx gene fused to the 5'

end of the HIV-1 tat gene, respectively.

Brief Description of the Drawings

The above objects, and other features and advantages of the present invention will become more apparent after a reading of the following detailed description when taken in conjunction with the drawings, in which:

Fig. 1 is a gene map of the vector pGX10 used to express the SIVmac239 immunogenic gene according to the present invention, wherein SV40 pA refers to SV40 polyA;

Fig. 2 is a gene map of the vector pGX10 (3.6 kb) used to express the SIVmac239 immunogenic gene according to the present invention;

Fig. 3 is a restriction map of the vector pGX10 (3.6kb) comprising 3641 nucleotides, which is used to express the SIVmac239 immunogenic gene according to the present invention, wherein the CMV promoter corresponds to nucleotides 3619-3641 and nucleotides 1 to 661; the TPL corresponds to nucleotides 666 to 1101; the SV40 late polyA sequence corresponds to nucleotides 1236 to 1457; the SV40 enhancer corresponds to nucleotides 1469 to 1713; the kanamycin resistance ORF corresponds to nucleotides 1727 to 2521; and the ColE1 origin corresponds to nucleotides 2907 to 3580, and the specific restriction sites are underlined;

Fig. 4 is a restriction map of the SIVmac239 clone;

Fig. 5 is a construction map of pGX10-SIV/GE according to the present invention, which is used as the SIVmac239 immunogenic plasmid;

Fig. 6 is a construction map of pGX10-SIV/dpol according to the present

invention, which is used as the SIVmac239 immunogenic plasmid;

Fig. 7 is a construction map of pGX10-SIV/VN according to the present invention, which is used as the SIVmac239 immunogenic plasmid;

Fig. 8 is a construction map of pGX10-SIV/TV according to the present invention, which is used as the SIVmac239 immunogenic plasmid;

Fig. 9 is a construction map of pGX10-SIV/VNTV according to the present invention, which is used as the SIVmac239 immunogenic plasmid;

Fig. 10 is a construction map of pGX10-SIV/TVVN according to the present invention, which is used as the SIVmac239 immunogenic plasmid;

Fig. 11 is a construction map of pGX10-HIV/GE according to the present invention, which is used as the HIV-1 immunogenic plasmid;

Fig. 12 is a construction map of pGX10-HIV/dpol according to the present invention, which is used as the HIV-1 immunogenic plasmid;

Fig. 13 is a construction map of pGX10-HIV/VN according to the present invention, which is used as the HIV-1 immunogenic plasmid;

Fig. 14 is a construction map of pGX10-HIV/TV according to the present invention, which is used as the HIV-1 immunogenic plasmid;

Fig. 15 is a construction map of pGX10-HIV/VNTV according to the present invention, which is used as the HIV-1 immunogenic plasmid;

Fig. 16 is a construction map of pGX10-HIV/TVVN according to the present invention, which is used as the HIV-1 immunogenic plasmid;

Fig. 17 is a construction map of the adjuvant plasmid pGX10-hp35/IRES/hp40 (pGX10-hIL-12m) according to the present invention, which is used as an adjuvant for the AIDS DNA vaccine of the present invention;

Fig. 18 is a graph showing expression levels of the human growth hormone expressed by the vector pGX10, which is used to express the SIVmac239 immunogenic gene according to the present invention, and by the conventional vectors pTV2 and pGX1 which are used as controls;

Fig. 19 is a construction map of the plasmid pTV2/hGH, which is used as a control for comparison of levels of the human growth hormone expressed by the vector pGX10, which is used to express the SIVmac239 immunogenic gene according to the present invention;

Fig. 20 is a construction map of the plasmid pGX1/hGH, which is used as a control for comparison of levels of the human growth hormone expressed by the vector pGX10, which is used to express the SIVmac239 immunogenic gene according to the present invention;

Fig. 21 is a restriction map of the adjuvant plasmid pAGGSIL-4, which is used as an adjuvant for the AIDS DNA vaccine according to the present invention;

Fig. 22 is a restriction map of the adjuvant plasmid pCAGGSIL-12, which is used as an adjuvant for the AIDS DNA vaccine according to the present invention;

Fig. 23 is a construction map of the plasmid pGX0/hGH, which is used as a control for comparison of abilities to induce immune response, as evaluated by expression of the human growth hormone, with the vector pGX10, which is used to express the SIVmac239 immunogenic gene according to the present invention;

Fig. 24 is a construction map of the plasmid pGX10/hGH, which is used as a control for comparison of abilities to induce immune response, through expression of the human growth hormone, with the vector pGX10, which is used to express the SIVmac239 immunogenic gene according to the present invention;

Fig. 25 is a graph showing the abilities to induce immune response, through expression of human growth hormone, of the vector pGX10, which is used to express the SIVmac239 immunogenic gene according to the present invention, and the plasmid pTV2/hGH and pGX0/hGH, which are used as controls;

Fig. 26 is a experimental protocol for evaluating the vaccine efficiencies of the immunogenic plasmids according to the present invention;

Fig. 27 is a graph showing the vacine efficiencies of the immunogenic plasmids according to the present invention, evaluated by counting the number of peripheral blood mononuclear cells (PBMC) in blood of Rhesus monkeys infected with SIVmac239. The results are expressed as the number of infectious PBMC per one million PBMC in blood of the treated monkey at various points of time after infection of the monkeys with SIVmac239. The X axis represents time (weeks) elapsing after infection with SIVmac239, the Y axis represents the number of infectious PBMC, and the 4 to 5 digit numbers represent the assigned numbers of respective monkeys;

Fig. 28 is a graph showing the vaccine efficiencies of the immunogenic plasmids according to the present invention, evaluated by counting copies of SIV RNA in blood plasma of Rhesus monkeys infected with SIVmac239. The results are expressed as the titers of SIV RNA detected in 1ml of the blood plasma of the treated monkeys at various points of time after infection of the monkeys with SIVmac239. The X axis represents time (weeks) elapsing after infection with SIVmac239, the Y axis represents the number of SIVmac239 RNA molecules per 1ml of the blood plasma, and the 4 to 5 digit numbers represent the assigned numbers of respective monkeys;

Fig. 29 is a graph showing the vaccine efficiencies of the immunogenic plasmids according to the present invention, evaluated by measuring the number of

absolute CD4+ cells in blood of Rhesus monkeys infected with SIVmac239. The results are expressed as percentages of the absolute number of CD4+ cells per unit volume of blood, relative to the absolute number of CD4+ cells per unit volume of blood before infection in the treated monkeys at various points of time after infection of the monkeys with SIVmac239. The X axis represents time (week) elapsing after infection with SIVmac239, the Y axis represents the percentages of the number of CD4+ cells in 1 $\mu\ell$ of blood relative to the CD4+ cells in 1 $\mu\ell$ of blood before infection in the treated monkeys at various points of time, and the 4 to 5 digit numbers represent the assigned numbers of respective monkeys;

Fig. 30 is a graph showing the vaccine efficiencies of the immunogenic plasmids according to the present invention, evaluated by measuring gag-specific T-cell response induced by SIV DNA immunization. The results are obtained by measuring the T-cell immune response induced by immunization in monkeys undergoing respective treatments just before infection with SIVmac239. The X axis represents the assigned numbers of respective monkeys, and the Y axis represents the number of cells secreting IFN-γ in response to stimulation by the gag peptide per one million PBMC;

Fig. 31 is a view showing the results of a Western blot analysis in which the plasmids pGX10-SIV/GE and pGX10-SIV/dpol according to the present invention, and the immunogenic plasmid pTV2-SIV/GE as a control, were transfected into HeLa cells and examined for the expression of antigen proteins by immunoblotting;

Fig. 32 is a view showing the results of a Western blot analysis in which the plasmids pGX10-SIV/GE and pGX10-SIV/dpol according to the present invention, and the immunogenic plasmid pTV2-SIV/GE and pTV2-SIV/dpol as controls, were

transfected into HeLa cells and examined for the expression of antigen proteins by immunoblotting;

Fig. 33 is a view showing the results of a Western blot analysis in which the plasmids pGX10-SIV/VN and pGX10-SIV/VNTV according to the present invention were transfected into HeLa cells and examined for the expression of adjuvant antigen proteins (Vif-Nef) by immunoblotting; and

Fig. 34 is a view showing the results of a Western blot analysis in which the plasmid pGX10-SIV/TV according to the present invention was transfected into HeLa cells and examined for the expression of adjuvant antigen protein (Tat-Vpx) by immunoblotting.

Best Mode for Carrying Out the Invention

As described above, there are many reports describing protective effects of vaccines against AIDS virus in monkeys. In order to determine the efficacy of a vaccine substance in a primate model, various species of monkeys are infected with various viruses and examined for protective effects of the vaccine. Here, the model can be diverse according to the species of the used non-human primates (chimpanzee, monkey) and the types of infecting virus (HIV-1, HIV-2, SHIV, SIVmne, SIVmac). Some models readily induce protection, while some models using a certain combination of monkeys and virus do not induce AIDS. Thus, these models can be classified into various types according to (1) the levels of virulence and severity of disease conditions, and (2) levels of induced protection, and efficacy of vaccines in the above various models are now carefully studied.

A representative model known to induce AIDS in monkey is a model in which a monkey is infected with SHIV89.6P. However, this model (1) uses virus which is produced by artificial recombination; (2) induces abnormally rapid decline in CD4 levels, thereby leading to death; (3) shows conditions by infection itself as well as symptoms of an immune deficiency disease condition; and (4) readily induces protection, as compared to the SIVmac239/monkey, as observed from many cases achieving successful protection using attenuated virus, DNA and recombinant virus vector, though it has been very recently developed, unlike SIVmac239. However, when using an attenuated virus as a vaccine, there are safety problems, since the attenuated virus can transform into a pathogenic virus. As another example, it has been attempted to induce protection through immunization with DNA and infection of blood with the virus SIVmac251. However, this attempt failed to induce protection, which caused reduction of CD4 levels in monkey, leading to death.

The present invention uses a model in which a DNA vaccine is administered to Rhesus macaques, which is then blood-infected with SIVmac239 to determine whether the vaccine can protect the monkey against the virus. The virus SIVmac239 is obtained by subjecting the virus SIVmac251 twice to *in vivo* passage, and hence has very similar base sequence, virulence and pathology to SIVmac251. There is no DNA vaccine which shows protective effects against blood infection with the virus SIVmac239. This model is characterized in that it can induce AIDS and also has an infection route, immunological indices after infection (CD4 number, CD29+CD4+T cell), and time to reach the maximum virus titer, which are all similar to the HIV-1 infection process in human beings. For this reason, the model used in the present invention is used in studies to determine the natures of protective immunization against

HIV-1 in human beings, i.e. to determine which immune response should be induced to protect against the infection by HIV-1. Defects of this model are rapid development of AIDS and inevitable death. However, the rapid development can be an advantage of the model in that results can be obtained in a short time. Another feature of this model is that it does not readily induce protection, as seen from the fact that only an immunization method using a vaccine of attenuated virus has succeeded in protection.

A DNA vaccine has been developed, which can successfully protect against the blood infection with the virus SIVmac239. In vaccine fields, "successful protection" means that, upon infection with virus after administration of a vaccine, one of the following conditions is observed: (1) no proliferation of the virus is observed and the virus is removed (sterilizing immunity has been induced); (2) proliferation of the virus is observed in the early infection stage but removed later (without development of any disease); (3) proliferation of the virus is suppressed for a long period of time and no disease is developed (no infection); and (4) the patient slowly develops a disease condition while maintaining the virus titer at a low level, thereby preventing infection.

It is believed that the reason that the DNA vaccine of the present invention can succeed in protection against blood infection of the virus SIVmac239 is by virtue of development of an excellent vaccine vector, codon optimization and effective use of adjuvant (regulatory) gene. The vector pGX10 which is developed as one aspect of the present invention is formed by augmenting the vector pTV2, which has been previously developed and filed by the present inventors, and the vector pTX, which has been disclosed by Lee A.H. et al. (Vaccine 1999, 17: 473-9). This vector has been proven to have a high level of expression in vitro (10 times higher than the vector pTV2) and also to show excellent immune response *in vivo* (inducing 10 times more

antibody response than the vector pTX). As for the immunogenic plasmid which is used in the DNA vaccine according to the present invention, the gene pol and adjuvant (regulatory) gene are fused with the DNA sequence encoding a signal peptide of secretory protein for codon optimization. The signal sequence of the secretory protein, for example, a signal sequence of gD (glycoprotein D) of Herpes Simplex virus has been shown to increase an expression level of a gene which has been fused thereto and immune response, particularly cell immune response, *in vivo* (Lee et al., J. Virol 72(10), 8430-36 (1998); in a DNA immunization using HCV structural gene, an increase of CTL responses were observed, as compared to immunization of ST DNA) Thus, in addition to the genes gag, pol and env, which have been conventionally used, adjuvant genes vif, nef, tat and vpx are used in the immunization. The gene vpr is not used as an adjuvant gene, since it is known to have an immune inhibitory effect (Ayyavoo Y., et al., Int Immunol., 14(1), 13-22 (2002)). Meanwhile, the genes nef and tat have not been used, due to their immune disturbance effects. Therefore, in the present invention, the genes nef and tat are fused to other adjuvant genes vif and vpx, respectively to produce expression vectors, so that their immune disturbance activities can be reduced. Also, where appropriate, not the full-length nef and tat genes, but a portion of them is used.

Definition of terminology

All the technically and scientifically related terminologies, which are included in the specification but are not defined, have meanings commonly accepted in the pertinent field of the present invention. Some of them are herein after defined in order to make clear their meanings, as follows:

The term "vector" as used herein refers to a DNA molecule which acts as a carrier which can safely deliver a foreign gene into a host cell. Also, in order to be a useful vector, it should be replicable and should have a device by which it can be introduced into the host cell, and there should be provided means of detecting its presence. Here, examples of the foreign gene include structural and adjuvant genes of SIVmac239 and HIV.

The term "plasmid" as used herein generally refers to a circular DNA molecule in which a foreign gene is operably linked to a vector so as to be expressed in a host cell. However, a plasmid can be a vector in that it is used to carry a foreign gene by treatment of certain restriction enzymes. Therefore, in this application, the terms plasmid and vector are interchangeably used, but may be distinguished in their meanings by those having ordinary knowledge in the genetic engineering field without clarification of their meanings.

The term "immunogenic plasmid" as used herein refers to a circular DNA molecule which includs a gene encoding an antigen and induces antigen-specific humoral and cell-mediated immune responses.

The term "adjuvant plasmid" as used herein refers to a circular DNA molecule which expresses an immunoregulatory molecule to promote antigen-specific humoral and cell-mediated immune responses induced by an immunogenic plasmid.

The term "structural gene" as used herein refers to gag, env and pol genes coding for structural proteins of SIVmac239 and HIV-1. The gag gene produces proteins having molecular weights of 55,000 (p55) daltons, 24,000 (p24) daltons, 17,000 (p17) daltons and 15,000 (p15) daltons. The p55 antigen is a precursor which is formed in the early stage of infection and then divided into different core proteins.

The gag protein exists in an inner nucleocapsid of virus. The p17 protein forms the matrix between the core and envelope and is buried in an inner part of the envelope. The p24 and p15 proteins form the core coats enclosing the nucleic acids. The env gene produces glycoproteins having molecular weights of 160,000 (gp160) daltons, 120,000 (gp120) daltons and 41,000 (gp41) daltons. The gp160 is a precursor of the glycoproteins gp120 and gp41 and is not a constitutional component of mature virus. The gp41 protein exists between the inner membrane and outer membrane and therefore, is also called a transmembrane protein. The gp120 protein forms 72 nobs over the envelope. The gp41 protein is involved in binding to CD4 molecule of a host cell, together with the gp120. The pol gene produces p66, p51 (reverse transcriptase), and p31 (integrase or endonuclease) proteins. The polymerase component plays a role in reverse-transcription of RNA to DNA and integration of DNA into cellular DNA, and functions to cleave a precursor into smaller active materials. The polymerase antigen exists within the core, in connection with nucleic acids. The gp160 and p55 proteins are precursors, which are secreted to blood during replication of the virus to produce antibodies, whereby they can be used to detect antibodies against these precursors by a serological method. In the present invention, the first immunogenic plasmid construct includes the nucleotide sequence corresponding to the protease coding part (not the full-length sequence) of the structural gene pol, which is expressed herein as "dpol". Also, the second immunogenic plasmid construct includes only the nucleotide sequence encoding reverse-transcriptase and integrase (not the full-length sequence) of the structural gene pol, which is expressed herein as "RT-INT". The RT-INT coding part can be optionally mutated.

The term "regulatory gene" or "adjuvant gene" as used herein refers to the nef,

vpr, vpu, tat, rev and vif genes, which encode regulatory proteins of SIVmac239 and HIV-1. Products of these regulatory genes function to modify expression of viral proteins and replication of virus, and regulate infectivity of the virus. Although exact roles of these genes are not yet known, it has been found that tat (p14) has transcription activity, rev (p19/20) regulates expression of virus mRNA, nef (p27) has various functions such as inhibition of CD4 receptor and regulation of T cell activity, vif (p23) increses infectivity of the virus, vpr (p15) supports replication of virus, vpu (p16) is involved in release of virus and vpx (p15) affects infectivity of the virus. However, in the present invention, vpr among the foregoing regulatory genes, is not used.

The term "operably linked" as used herein means that the respective components of a plasmid or vector are arranged so as to exert their own functions. Therefore, a control sequence operably linked to a coding sequence can affect expression of the coding sequence. The control sequence does not need to lie adjacent to the coding sequence as long as the control sequence can act to regulate the expression of the coding sequence. For example, when an intervening sequence is disposed between a promoter sequence and a coding sequence, the promoter sequence can be said to be "operably linked" to the coding sequence.

Vector pGX10

The present inventors developed a basic vector for an immunogenic plasmid to be contained in an AIDS DNA vaccine, and designated the pGX10. As shown in Fig. 2 and Fig. 3, the vector pGX10 of the invention is a novel vector of 3.6 kb, characterized by comprising SV40 ori, simian virus 40 replication origin, cytomegalovirus (CMV) early promoter/enhancer sequence, adenovirus tripartite leader

sequence (TPL), multi-cloning site (MCS), simian virus 40 polyadenylation sequence (SV40PA), simian virus 40 enhancer sequence (SV40Eh), ColE1 Ori and a kanamycin resistance gene. This vector can proliferate in E. coli, and has a plurality of particular restriction sites.

The vector pGX10 was prepared from a known vector, that is pTX, which had been previously disclosed by the present inventors (Lee A.H., et al., Vaccine 17:473-9 (1999)), as described and illustrated in Example 1 and Fig. 1, and can be prepared using pTV2 vector, which has been used as a DNA vaccine vector in studies on small animals (Lee, et al., J Virol. 72,8430 (1998); and Cho, et al., Vaccine 17,1136 (1999)), as a starting vector according to a known method. The vector pGX10 was deposited with Korean Collection of Type Cultures (KCTC), one of international depository authorities, on March, 2002, as Accession No. KCTC 10212BP, under the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure. It is apparent to those skilled in the art that types of the promoter and types and lengths of the glycoprotein signal sequence can be changed in various ways depending on the purpose for practicing the present invention. For example, the promoters which can be used include viral promoters such as RSV promoter, cellular promoters such as EF1, MCK (muscle specific promoter), LCK (T cell specific promoter). For the glycoprotein, VZV (varicella zoster virus) gB, HCMV (human cytomegalovirus) gH, gL, GO, VSV (vesicular stomatitis virus) G protein, rotavirus outer capsid glycoprotein), VP7 can be substituted.

The vector pGX10 is an improvement of the vector pTX and pTV2, which are already known, as described above, and shows a high level of expression in vitro (10 times higher than pTV2, Fig. 18) and moreover, generates a superior immune response,

even *in vivo*. It was observed that the vector pGX10 induces immune responses at a level 10 times stronger than pTX, and pGX10-HIV/RT (reverse transcriptase) produces anti-RT antibodies in an amount 10 times greater than pTX10-HIV/RT (data not shown). The known vector pTV2 is disclosed in Korean Patent Application Laid-open No. 2001-0054338(July 2, 2001) and its corresponding US Patent Publication No. 2001004531 (June 21, 2001).

SIV immunogenic plasmids

According to the present invention, the following four types of basic immunogenic plasmids were constructed for use as AIDS DNA vaccines to be examined for their efficacy in rhesus macaques monkeys:

1) An immunogenic plasmid (hereinafter referred to as "first SIV immunogenic plasmid) comprising: the vector pGX10, and (i) the SIVmac239 gag gene encoding matrix protein (MA), capsid protein (CA) and nucleocapsid protein (NC); (ii) the SIVmac239 dpol sequence in the pol gene, encoding protease; (iii) the SIVmac239 env gene encoding envelope protein; and (iv) the SIVmac239 regulatory gene rev, encoding the protein Rev, operably linked thereto;

2) An immunogenic plasmid (hereinafter referred to as "second SIV immunogenic plasmid) comprising: the vector pGX10, and the SIVmac239 pol gene encoding reverse transcriptase (RT) and integrase (INT) and a DNA sequence encoding a signal peptide of secretory protein fused to the 3' end of the SIVmac239 pol gene, operably linked thereto;

3) An immunogenic plasmid (hereinafter referred to as "third SIV immunogenic plasmid) comprising: the SIVmac239 vif gene, and a DNA sequence

encoding signal peptide of secretory protein and the SIVmac239 nef gene, fused to the 3' and 5' ends of the SIVmac239 vif gene, respectively; and

4) An immunogenic plasmid (hereinafter referred to as "fourth SIV immunogenic plasmid) comprising: a DNA sequence comprising any one of genes having from exon 1 to a full-length sequence of the SIVmac239 tat gene, and a DNA sequence encoding signal peptide of secretory protein and the SIVmac239 vpx gene fused to the 3' and 5' ends of the SIVmac239 tat gene, respectively.

Now, the above four types of immunogenic plasmids will be described in detail.

(1) First SIV immunogenic plasmid

This novel immunogenic plasmid of 8.7 kb is formed by introducing the gag, dpol (protease) and env genes, and the SIVmac239 rev regulatory gene to be operably linked to the MCS (multi-cloning site) of the vector pGX10 according to the present invention, which is used in the DNA vaccine against the SIVmac239/rhesus macaques monkey according to the present invention. The detailed procedure for constructing this plasmid is described in Example 2 and illustrated Fig. 5. This plasmid is designated pGX10-SIV/GE and was deposited with Korean Collection of Type Cultures (KCTC), one of international depository authorities, on March, 2002, as Accession No. KCTC 10215BP, under the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure.

The plasmid pGX10-SIV/GE shows a superior expression efficiency, as compared to the immunogenic plasmid pTV-SIV/GE of 10.0 kb disclosed in pending Korean Patent Application Laid-open No. 2001-0054338 and its corresponding US Patent Publication No. 2001004531, which were filed by the present inventors. This

can be confirmed by Fig. 31 showing a photograph of Western blotting analyses of pGX10-SIV/GE and pTV-SIV/GE.

(2) Second SIV immunogenic plasmid

This novel immunogenic plasmid is formed by introducing the SIVmac239 pol gene encoding reverse transcriptase and integrase and the DNA sequence encoding signal peptide of secretory protein fused to the 3' end of the SIVmac239 pol gene into the MCS (multi-cloning site) to be operably linked to the vector pGX10 according to the present invention, which is used in the DNA vaccine against the SIVmac239/rhesus macaques monkey according to the present invention, together with the first SIV immunogenic plasmid.

In a preferred embodiment of the present invention, the pol gene may be mutated so that its integrase activity is suppressed. When using such mutated gene in a DNA vaccine, possibility of production of a virus which can proliferate in the subject inoculated with the DNA vaccine is further lowered, thereby leading to improvement in safety. For example, nucleotides 5130-5135 site in the integrase region is known to be very important for the enzyme activity of integrase (codon for Asp 116, Fields Virology, Third edition p1893, Lippincott-Raven Co., 1996). Therefore, it is possible to inhibit the activity of integrase by modifying nucleotides 5130-5135 site so as to prevent proliferation of the virus in host cells. Particularly, nucleotides 5130-5132 site of integrase is deleted and/or nucleotides 5133-5135 site is substituted with codon for serine. Consequently, the gene was mutated to express Ser117, instead of Asn117. In our own experiments, it was confirmed that such mutated SIVmac239 virus did not proliferate in host cells. The above-described position numbers of the base sequence

followed the SIVmac239 clone of GeneBank Accession Number M33262 (Fig. 4).

In addition, it is possible to inhibit the activity of integrase by modifying coding sequences in which Asp116, Asp64 and Glu152 are conserved. These amino acids are known to stabilize transition state by coupling with a divalent metal ion such as Mg^{2+} at the active site of the integrase. For HIV-1 integrase, His12, Hix16, Cys40 and Cys43 are important amino acids to form DNA binding structure (metal-finger)(Field Virology supra, p1893).

To the 3' end of the SIVmac239 pol gene ecoding reverse transcriptase and integrase (expressed as RT-INT in Fig. 6), a DNA sequence encoding signal peptide of secretory protein is fused. As a result of such fusion, transcription of transcriptase and integrase is directly controlled by CMV promoter, thereby increasing expression levels of the enzymes. Preferably, a signal peptide of glycoprotein is used as a signal sequence of secretory protein. Examples of the glycoprotein include herpes simplex virus glycoprotein gD, varicella zoster virus (VZV) gB, human cytomegalovirus (HCMV) gH, gL, gO, vesicular stomatitis virus (VSV) G protein, rotavirus outer capsid glycoprotein, VP7 and the like.

In a preferred embodiment, a second immunogenic plasmid of 6.3 kb is formed by inserting the SIVmac239 pol gene encoding reverse transcriptase and integrase in which the nucleotides 5130-5132 site is deleted and the nucleotides 5133-5135 site is substituted with serine codon, and a DNA sequence encoding a signal peptide of glycoprotein D (gD) derived from herpes simplex virus (HSV) which is fused to the 3' end of the SIVmac239 pol gene, into the MCS (multi-cloning site) to be operably linked to the vector pGX10 according to the present invention. The pol gene comes under direct transcriptional control of CMV promoter due to the DNA signal sequence

encoding 33 N-terminal amino acids of HSV gD, which is fused to the 3' end of the pol gene, thereby increasing expression strength of reverse transcriptase and integrase. This plasmid is used as a second SIV immunogenic plasmid of 8.7 kb in the DNA vaccine against the SIVmac239/rhesus macaques monkey according to the present invention. The detailed procedure for constructing this plasmid is described in Example 3 and illustrated Fig. 6. This plasmid is designated pGX-SIV/dpol and was also deposited with Korean Collection of Type Cultures (KCTC), one of international depository authorities, on March, 2002, as Accession No. KCTC 10214BP, under the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure.

Meanwhile, it should be understood that various changes and modifications of methods for mutagenesis, types of promoter, and types and lengths of the glycoprotein signal sequence, other than those described above, can be made according to the purpose for practicing the present invention, and such changes and modifications will be apparent to those skilled in the art.

The plasmid pGX10-SIV/dpol shows a superior expression efficiency (data not shown), as compared to the immunogenic plasmid pTV-SIV/dpol of 10.0 kb disclosed in Korean Patent Application Laid-open No. 2001-0054338 and its corresponding US Patent Publication No. 2001004531, which were filed by the present inventors.

(3) Third SIV immunogenic plasmid

This novel plasmid is constructed by inserting the SIVmac239 vif gene, and a DNA sequence encoding a signal peptide of secretory protein and the SIVmac239 nef gene fused to the 3' and 5' ends of the SIVmac239 vif gene, respectively, to a vector, in which the genes and signal sequence are operably linked to the vector. The vector

which can be used includes any mammalian cell expression vectors, preferably, a DNA vaccine vector optimized to induce immune response upon expression in muscle cells, DC (dendritic cells), and T cells. More preferably, the vector is a vector including CMV promoter and optionally TPL sequence SV40 pA. Concrete examples of vectors which can be used in the present invention include, but are not limited to, pGX10 and pTV2; pVX1, pRC/CMV, pREP4 and pREP10 (though including RSV promoter), pcDNA1, pcDNA1.1, pcDNA3, pcDNA3/CAT, pRC/CMV and pRC/CMV2 supplied by Invitrogen Corp.; pCMV-script supplied by Stratagene; and pSI, pCI and pCI-neo supplied by Promega. The most preferred is pGX10.

The third immunogenic plasmid thus constructed is delivered along with the first SIV immunogenic plasmid pGX10-SIV/GE and the second SIV immunogenic plasmid (for example, pGX10-SIV/dpol) to enhance the protection induced by the first and second immunogenic plasmids.

In addition, the third immunogenic plasmid is delivered along with the first SIV immunogenic plasmid pGX10-SIV/GE and the second SIV immunogenic plasmid (for example, pGX10-SIV/pol) and the fourth SIV immunogenic plasmid (for example, pGX10-SIV/TV) to enhance the protection induced by the first and second immunogenic plasmids.

In a preferred embodiment of the present invention, the SIVmac239 vif regulatory gene and the SIVmac239 nef regulatory gene, which is fused to the 5' end of the SIVmac239 vif regulatory gene, may be modified to remove immunosuppressive effects. The modification can be effected by various methods. For example, Ser114-Leu150 in the vif gene can be modified (Fields Virology Third edition, p1901, Lippincott-Raven Col, 1996) and Arg137, Arg138 and Gly2 (involved in myristylation)

in the nef gene can be modified.

To the 3' end of the SIVmac239 vif gene, a DNA sequence encoding a signal peptide of secretory protein is fused. As a result of such fusion, the transcription of the vif gene is directly controlled by CMV promoter, thereby increasing expression levels of the Vif and Nef proteins. Preferably, the DNA sequence encoding a signal peptide of glycoprotein is used as the DNA sequence encoding a signal peptide of secretory protein. Examples of the glycoprotein include herpes simplex virus glycoprotein gD, varicella zoster virus (VZV) gB, human cytomegalovirus (HCMV) gH, gL, gO, vesicular stomatitis virus (VSV) G protein, rotavirus outer capsid glycoprotein, VP7 and the like.

In one embodiment, another immunogenic plasmid of 5.1 kb is constructed by inserting the SIVmac239 vif gene, and a DNA sequence encoding a signal peptide of HSV gD and the modified SIVmac239 nef gene fused to the 3' and 5' ends of the SIVmac239 vif gene, respectively, to the MCS (multi-cloning site) of the vector pGX10 according to the present invention, in which the genes and the signal sequence are operably linked to the vector. This plasmid is used as a third or fourth SIV immunogenic plasmid in the DNA vaccine against the SIVmac239/rhesus macaques monkey according to the present invention. The detailed procedure for constructing this plasmid is described in Example 4 and illustrated Fig. 7. This plasmid is designated pGX-SIV/VN and was also deposited with Korean Collection of Type Cultures (KCTC), one of international depository authorities, on March, 2002, as Accession No. KCTC 10213BP, under the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure.

To be concrete, the third SIV immunogenic plasmid pGX10-SIV/VN

comprises a gene (VN) formed by binding the SIVmac239 vif and nef. The nef gene is modified by the deletion of codons for Arg137 and Arg138 which are known to play an important role in the downregulation activity of CD4 (J. Biol. Chem. 270; 15307, 1995) so as to prevent the immunosuppressive effects of the Nef protein. Thus, this plasmid was devised to increase expression of fused Vif-Nef by fusing the signal sequence encoding 33 N-terminal amino acids of HSV gD to the 3' end of the VN gene so that the VN gene comes under direct transcription control of CMV promoter.

Meanwhile, it should be understood that various changes and modifications of methods for mutagenesis, types of promoter, and types and lengths of the glycoprotein signal sequence, other than those described above, can be made according to the purpose for practicing the present invention, and such changes and modifications will be apparent to those skilled in the art.

(4) Forth SIV immunogenic plasmid

This novel plasmid is constructed by inserting a DNA sequence comprising any one of genes having from exon 1 to a full-length sequence of the SIVmac239 tat gene, and a DNA sequence encoding signal peptide of secretory protein and the SIVmac239 vpx gene fused to the 3' and 5' ends of the SIVmac239 tat gene, respectively, into a vector, in which the genes and signal sequence are operably linked to the vector. The vector which can be used includes any mammalian cell expression vectors, preferably, a DNA vaccine vector optimized to induce immune response upon expression in muscle cells, DC, and T cells. More preferably, the vector is a vector including CMV promoter and optionally TPL sequence SV40 pA. Concrete examples of vectors which can be used in the present invention include, but are not limited to,

pGX10 and pTV2; pVX1, pRC/CMV, pREP4 and pREP10 (though including RSV promoter), pcDNA1, pcDNA1.1, pcDNA3, pcDNA3/CAT, pRC/CMV and pRC/CMV2 supplied by Invitrogen Corp.; pCMV-script supplied by Stratagene; and pSI, pCI and pCI-neo supplied by Promega. The most preferred is pGX10.

The fourth plasmid thus constructed is delivered along with the first SIV immunogenic plasmid pGX10-SIV/GE and the second SIV immunogenic plasmid (for example, PGX10-SIV/dpol) to enhance the protection induced by the first and second immunogenic plasmids.

Additionally, the fourth SIV immunogeinc plasmid is delivered along with the first SIV immunogenic plasmid pGX10-SIV/GE, the second SIV immunogenic plasmid (for example, pGX10-SIV/dpol) and the third SIV immunogenic plasmid (for example, pGX10-SIV/VN) to enhance the protection induced by the first and second immunogenic plasmids.

The tat gene is preferably used in a modified form since it can be bring about suppression of immune responses in a immunized host. A region in the tat gene which can be modified comprises the entire gene except exon 1. The exon 1 of the tat gene expresses the enzyme activity (immune disturbance) of the Tat but the effect is less than that of exon 1+exon 2. The immune epitope included in exon 2 of the tat gene cannot be used. Therefore, it is the most preferable to use only the exon 1 site of the tat gene.

To the 3' end of the SIVmac239 tat regulatory gene, a DNA sequence encoding a signal peptide of secretory protein is fused. As a result of such fusion, the transcription of the tat gene is directly controlled by the CMV promoter, thereby increasing expression levels of the Tat and Vpx proteins. Preferably, the DNA

sequence encoding a signal peptide of glycoprotein is used as the DNA sequence encoding a signal peptide of secretory protein. Examples of the glycoprotein include herpes simplex virus glycoprotein gD, varicella zoster virus (VZV) gB, human cytomegalovirus (HCMV) gH, gL, gO, vesicular stomatitis virus (VSV) G protein, rotavirus outer capsid glycoprotein, VP7 and the like.

In a preferred embodiment, another immunogenic plasmid of 4.3 kb is constructed by inserting exon 1 of the SIVmac239 tat gene, and a DNA sequence encoding a signal peptide of HSV gD and the SIVmac239 vpx gene fused to the 3' and 5' ends of the SIVmac239 tat gene, respectively, into the MCS (multi-cloning site) of the vector pGX10 according to the present invention, in which the genes and the signal sequences are operably linked to the vector. This plasmid is used as a third or fourth SIV immunogenic plasmid in the DNA vaccine against the SIVmac239/rhesus macaques monkey according to the present invention. The detailed procedure for constructing this plasmid is described in Example 5 and illustrated Fig. 8. This plasmid is designated pGX-SIV/TV and was also deposited with Korean Collection of Type Cultures (KCTC), one of international depository authorities, on April, 2002, as Accession No. KCTC 10216BP, under the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure.

To be concrete, the fourth SIV immunogenic plasmid pGX10-SIV/TV comprises a gene (TV) formed by fusing exon 1 of the SIVmac239 tat gene and vpx gene. Thus, this plasmid was formed by fusing the signal sequence encoding 33 N-terminal amino acids of HSV gD to the 5' end of the TV gene so that the gene TV comes under direct transcriptional control of CMV promoter.

Meanwhile, it should be understood that various changes and modifications of

methods for mutagenesis, types of promoter, and types and lengths of the glycoprotein signal sequence, other than those described above, can be made according to the purpose for practicing the present invention, and such changes and modifications will be apparent to those skilled in the art.

(5) Immunogenic plasmid comprising the SIVmac239 regulatory genes vif, nef, tat and vpx

In a preferred embodiment of the present invention, a plasmid is constructed by inserting (i) the SIVmac239 vif gene, and a DNA sequence encoding a signal peptide of secretory protein and the SIVmac239 nef gene fused to the 3' and 5' ends of the SIVmac239 vif gene, respectively, and (ii) a DNA sequence comprising any one of genes having from exon 1 to a full-length sequence of the SIVmac239 tat gene, and a DNA sequence encoding a signal peptide of secretory protein and the SIVmac239 vpx gene fused to the 3' and 5' ends of the SIVmac239 tat gene, respectively, into a vector, in which the genes and signal sequences are operably linked to the vector. Concrete examples of the vector which can be used in the present invention include, but are not limited to, pGX10 and pTV2; pVX1, pRC/CMV, pREP4 and pREP10 (though including RSV promoter), pcDNA1, pcDNA1.1, pcDNA3, pcDNA3/CAT, pRC/CMV and pRC/CMV2 supplied by Invitrogen Corp.; pCMV-script supplied by Stratagene; and pSI, pCI and pCI-neo supplied by Promega. The most preferred is pGX10.

The plasmid thus constructed is delivered along with the first SIV immunogenic plasmid pGX10-SIV/GE and the second SIV immunogenic plasmid (for example, pGX10-SIV/dpol) to enhance the protection induced by the first and second immunogenic plasmids. Also, this plasmid can reduce the number of immunogenic

plasmid which should be prepared for a DNA vaccine, thereby lowering the production cost.

In a preferred embodiment of the present invention, the SIVmac239 vif regulatory gene and the SIVmac239 nef regulatory gene, which is fused at the 5' end of the SIVmac239 vif regulatory gene, may be modified to remove its immunosuppressive effects. The modification can be effected by various methods. For example, Ser114-Leu150 in the gene vif can be modified (Fields Virology Third edition, p1901, Lippincott-Raven Col, 1996) and Arg137, Arg138 and Gly2 (involved in myristylation) in the gene nef can be modified.

In another preferred embodiment of the present invention, the tat gene is preferably used in a modified form since it may bring about immune disturbance, though it can be used in its full-length form. A region in the tat gene which can be modified comprises the entire gene except exon 1. The exon 1 of the tat gene expresses the enzyme activity (immune disturbance) of the Tat but the effect is less than that of exon 1+exon 2. The immune epitope included in exon 2 of the tat gene cannot be used. Therefore, it is the most preferable to use only exon 1 site of the tat gene. The nef and tat genes can be independently modified. For example, there can be a case where the nef gene is modified while the tat gene is not modified, and where the tat gene is modified while the nef gene is not modified, or where both the nef and tat genes are modified.

To each 3' end of the SIVmac239 vif and tat genes, a DNA sequence encoding a signal peptide of secretory protein is fused. As a result of such fusion, the transcription of the vif and tat genes is directly controlled by each CMV promoter, thereby increasing expression levels of the Vif and Nef proteins. Preferably, the DNA

sequence encoding a signal peptide of glycoprotein is used as a signal sequence of secretory protein. Examples of the glycoprotein include herpes simplex virus glycoprotein gD, varicella zoster virus (VZV) gB, human cytomegalovirus (HCMV) gH, gL, gO, vesicular stomatitis virus (VSV) G protein, rotavirus outer capsid glycoprotein, VP7 and the like.

In one embodiment, an immunogenic plasmid of 7.5 kb is constructed by inserting exon 1 of the SIVmac239 tat gene, and a DNA sequence encoding signal peptide of HSV gD and the SIVmac239 vpx gene fused to the 3' and 5' ends of the SIVmac239 tat gene, respectively, into a third SIV immunogenic plasmid comprising the SIVmac239 vif gene, and a DNA sequence encoding a signal peptide of HSV gD and the modified SIVmac239 nef gene (having codons for Arg137 and Arg138 deleted) fused to the 3' and 5' ends of the SIVmac239 vif gene, respectively, to be operably linked to MCS (multi-cloning site) of the vector pGX10 according to the invention, in which the genes and the signal sequences are operably linked to the third SIV immunogenic plasmid. This plasmid is used as an additional SIV immunogenic plasmid in the DNA vaccine against the SIVmac239/rhesus macaques monkey according to the present invention. The detailed procedure for constructing this plasmid is described in Example 6 and illustrated Fig. 9. This plasmid is designated pGX-SIV/VNTV.

In another preferred embodiment, an immunogenic plasmid of 7.5 kb is constructed by inserting the SIVmac239 vif gene, and a DNA sequence encoding signal peptide of HSV gD and the modified SIVmac239 nef gene fused to the 3' and 5' ends of the SIVmac239 vif gene, respectively, into a fourth SIV immunogenic plasmid comprising exon 1 of the SIVmac239 tat gene, and a DNA sequence encoding signal

peptide of HSV gD and the SIVmac239 vpx gene fused to the 3' and 5' ends of the SIVmac239 tat gene, respectively, to be operably linked to MCS (multi-cloning site) of the vector pGX10 according to the present invention, in which the genes and the signal sequences are operably linked to the fourth SIV immunogenic plasmid. This plasmid is used as an additional SIV immunogenic plasmid in the DNA vaccine against the SIVmac239/rhesus macaques monkey according to the present invention. The detailed procedure for constructing this plasmid is described in Example 7 and illustrated Fig. 10. This plasmid is designated pGX-SIV/TVVN.

Meanwhile, it should be understood that various changes and modifications of methods for mutagenesis, types of promoter, and types and lengths of the glycoprotein signal sequence, other than those described above, can be made according to the purpose for practicing the present invention, and such changes and modifications will be apparent to those skilled in the art.

It is obvious the present invention is effective for treating HIV-1 infection in humans. HIV-1 and SIV both belong to lentivirus species, and structures and functions of their structural genes are not exactly the same, but are very similar. Also, within some genes commonly found in HIV-1 and SIV, such as the gag gene, the DNA sequences have a high homology and their polyclonal antibodies are cross-reactive. Above all, the conditions appearing in humans infected with HIV are similar to the conditions appearing in monkeys infected with SIV. For these reasons, when substituting SIV genes with HIV genes while using a human model, it may be expected to achieve efficacy similar to that in a SIV/monkey model. Of course, because no AIDS vaccine has been reported which succeeds in humans, it is not guaranteed that the assessment in SIVmac/monkey model will be perfectly replicated in a human model.

Since there may exist factors which act only on human beings, it is possible that a substance which are effective in primate models such as the SIVmac239/monkey model is not effective at all in humans. However, the present invention (using a primate model) can be effectively used to assess a candidate substance before testing the efficacy of a vaccine in human beings and thereby, to reduce the risk of the clinical demonstration. Moreover, it can be readily expected that a substance showing excellent effects in primate models has a higher possibility to be applied to human beings, as compared to a substance showing excellent effects in non-primate models such as mouse, cat, etc.

HIV immunogenic plasmids

According to the present invention, the following four types of basic immunogenic plasmids were constructed for use as AIDS DNA vaccines to be examined for their efficacy in humans:

1) An immunogenic plasmid (hereinafter referred to as "first HIV immunogenic plasmid) comprising: the vector pGX10, and (i) the HIV-1 gag gene encoding matrix protein (MA), capsid protein (CA) and nucleocapsid protein (NC); (ii) the HIV-1 dpol sequence in the pol gene, encoding protease; (iii) the HIV-1 env gene encoding envelope protein; and (iv) the HIV-1 regulatory gene rev, encoding the protein Rev, operably linked thereto;

2) An immunogenic plasmid (hereinafter referred to as "second HIV immunogenic plasmid) comprising: the vector pGX10, and the HIV-1 pol gene encoding reverse transcriptase (RT) and integrase (INT) and a DNA sequence encoding a signal peptide of secretory protein fused to the 3' end of the HIV-1 pol gene, operably

linked thereto;

3) An immunogenic plasmid (hereinafter referred to as "third HIV immunogenic plasmid) comprising: the HIV vif gene, and a DNA sequence encoding signal peptide of secretory protein and the HIV-1 nef gene, fused to the 3' and 5' ends of the HIV-1 vif gene, respectively; and

4) An immunogenic plasmid (hereinafter referred to as "fourth HIV immunogenic plasmid) comprising: a DNA sequence comprising any one of genes having from exon 1 to a full-length sequence of the HIV-1 tat gene, and a DNA sequence encoding signal peptide of secretory protein and the HIV-1 vpx gene fused to the 3' and 5' ends of the HIV-1 tat gene, respectively.

Now, the above four types of HIV immunogenic plasmids will be described in detail.

(1) First HIV immunogenic plasmid

This novel immunogenic plasmid of 8.7 kb is formed by introducing the gag, dpol (protease) and env genes, and the HIV rev regulatory gene to be operably linked to the MCS (multi-cloning site) of the vector pGX10 according to the present invention, which is used in the DNA vaccine against AIDS human patients. The detailed procedure for constructing this plasmid is described in Example 8 and illustrated Fig. 11. This plasmid is designated pGX10-HIV/GE.

The plasmid pGX10-SIV/GE shows a superior expression efficiency, as compared to the immunogenic plasmid pTV-HIV/GE of 11.0 kb disclosed in Korean Patent Application Laid-open No. 2001-0054338 and its corresponding US Patent Publication No. 2001004531, which were filed by the present inventors.

(2) Second HIV immunogenic plasmid

This novel immunogenic plasmid is formed by introducing the HIV pol gene encoding reverse transcriptase and integrase and the DNA sequence encoding signal peptide of secretory protein fused to the 3' end of the HIV pol gene into the MCS (multi-cloning site) to be operably linked to the vector pGX10 according to the present invention, which is used in the DNA vaccine against AIDS human patients, together with the first HIV immunogenic plasmid.

In a preferred embodiment of the present invention, the pol gene may be mutated so that its integrase activity is suppressed. When using such mutated gene in a DNA vaccine, possibility of production of a virus which can proliferate in the subject inoculated with the DNA vaccine is further lowered, thereby leading to improvement in safety. For example, nucleotides 5130-5135 site in the integrase region is known to be very important for the enzyme activity of integrase (codon for Asp 116, Fields Virology, Third edition p1893, Lippincott-Raven Co., 1996). Therefore, it is possible to inhibit the activity of integrase by modifying nucleotides 5130-5135 site so as to prevent proliferation of the virus in host cells. Particularly, nucleotides 5130-5132 site of integrase is deleted and/or nucleotides 5133-5135 site is substituted with codon for serine. Consequently, the gene was mutated to express Ser117, instead of Asn117. In our own experiments, it was confirmed that such mutated HIV-1 virus did not proliferate in host cells. The above-described position numbers of the nucleotide sequence followed the HIV-1 JR-CSF clone of GeneBank Accession Number M38429.

In addition, it is possible to inhibit the activity of integrase by modifying coding sequences in which Asp116, Asp64 and Glu152 are conserved. These amino

acids are known to stabilize transition state by coupling with a divalent metal ion such as Mg^{2+} at the active site of the integrase. For HIV-1 integrase, His12, Hix16, Cys40 and Cys43 are important amino acids to form DNA binding structure (metal-finger)(Field Virology supra, p1893).

To the 3' end of the HIV-1 pol gene encoding reverse transcriptase and integrase (expressed as RT-INT in the drawings), a DNA sequence encoding signal peptide of secretory protein is fused. As a result of such fusion, transcription of transcriptase and integrase is directly controlled by CMV promoter, thereby increasing expression levels of the enzymes. Preferably, a signal peptide of glycoprotein is used as a signal sequence of secretory protein. Examples of the glycoprotein include herpes simplex virus glycoprotein gD, varicella zoster virus (VZV) gB, human cytomegalovirus (HCMV) gH, gL, gO, vesicular stomatitis virus (VSV) G protein, rotavirus outer capsid glycoprotein, VP7 and the like.

In one embodiment, a second HIV immunogenic plasmid of 6.2 kb is formed by inserting the HIV pol gene encoding reverse transcriptase and integrase in which the nucleotides 5130-5132 site is deleted and the nucleotides 5133-5135 site is substituted with serine codon, and a DNA sequence encoding a signal peptide of glycoprotein D (gD) derived from herpes simplex virus (HSV) which is fused to the 3' end of the HIV-1 pol gene, into the MCS (multi-cloning site) to be operably linked to the vector pGX10 according to the present invention. The pol gene comes under direct transcriptional control of CMV promoter due to the DNA signal sequence encoding 33 N-terminal amino acids of HSV gD, which is fused to the 3' end of the pol gene, thereby increasing expression strength of reverse transcriptase and integrase. This plasmid is used as a second HIV immunogenic plasmid in the DNA vaccine against

AIDS human patients. The detailed procedure for constructing this plasmid is described in Example 9 and illustrated Fig. 12. This plasmid is designated pGX-HIV/dpol.

Meanwhile, it should be understood that various changes and modifications of methods for mutagenesis, types of promoter, and types and lengths of the glycoprotein signal sequence, other than those described above, can be made according to the purpose for practicing the present invention, and such changes and modifications will be apparent to those skilled in the art.

The plasmid pGX10-HIV/dpol shows a superior expression efficiency (data not shown), as compared to the immunogenic plasmid pTV-HIV/dpol of 7.5 kb disclosed in Korean Patent Application Laid-open No. 2001-0054338 and its corresponding US Patent Publication No. 2001004531, which were filed by the present inventors.

(3) Third SIV immunogenic plasmid

This novel plasmid is constructed by inserting the HIV-1 vif gene, and a DNA sequence encoding a signal peptide of secretory protein and the HIV-1 nef gene fused to the 3' and 5' ends of the HIV-1 vif gene, respectively, to a vector, in which the genes and signal sequence are operably linked to the vector. The vector which can be used includes any mammalian cell expression vectors, preferably, a DNA vaccine vector optimized to induce immune response upon expression in muscle cells, DC, and T cells. More preferably, the vector is a vector including CMV promoter and optionally TPL sequence SV40 pA. Concrete examples of vectors which can be used in the present invention include, but are not limited to, pGX10 and pTV2; pVX1, pRC/CMV, pREP4 and pREP10 (though including RSV promoter), pcDNA1, pcDNA1.1, pcDNA3,

pcDNA3/CAT, pRC/CMV and pRC/CMV2 supplied by Invitrogen Corp.; pCMV-script supplied by Stratagene; and pSI, pCI and pCI-neo supplied by Promega. The most preferred is pGX10.

The third immunogenic plasmid thus constructed is delivered along with the first SIV immunogenic plasmid pGX10-HIV/GE and the second HIV immunogenic plasmid (for example, pGX10-HIV/dpol) to enhance the protection induced by the first and second immunogenic plasmids.

In addition, the third immunogenic plasmid is delivered along with the first HIV immunogenic plasmid pGX10-HIV/GE and the second SIV immunogenic plasmid (for example, pGX10-HIV/pol) and the fourth HIV immunogenic plasmid (for example, pGX10-HIV/TV) to enhance the protection induced by the first and second immunogenic plasmids.

In a preferred embodiment of the present invention, the HIV-1 vif regulatory gene and the HIV-1 nef regulatory gene, which is fused to the 5' end of the HIV-1 vif regulatory gene, may be modified to remove immunosuppressive effects. The modification can be effected by various methods. For example, Ser114-Leu150 in the vif gene can be modified (Fields Virology Third edition, p1901, Lippincott-Raven Col, 1996) and Arg137, Arg138 and Gly2 (involved in myristylation) in the nef gene can be modified.

To the 3' end of the HIV vif gene, a DNA sequence encoding a signal peptide of secretory protein is fused. As a result of such fusion, the transcription of the vif gene is directly controlled by CMV promoter, thereby increasing expression levels of the Vif and Nef proteins. Preferably, the DNA sequence encoding a signal peptide of glycoprotein is used as the DNA sequence encoding a signal peptide of secretory

protein. Examples of the glycoprotein include herpes simplex virus glycoprotein gD, varicella zoster virus (VZV) gB, human cytomegalovirus (HCMV) gH, gL, gO, vesicular stomatitis virus (VSV) G protein, rotavirus outer capsid glycoprotein, VP7 and the like.

In one embodiment, another immunogenic plasmid of 5.1 kb is constructed by inserting the HIV-1 vif gene, and a DNA sequence encoding a signal peptide of HSV gD and the modified HIV-1 nef gene fused to the 3' and 5' ends of the SIVmac239 vif gene, respectively, to the MCS (multi-cloning site) of the vector pGX10 according to the present invention, in which the genes and the signal sequence are operably linked to the vector. This plasmid is used as a third or fourth HIV immunogenic plasmid in the DNA vaccine against AIDS human patients. The detailed procedure for constructing this plasmid is described in Example 10 and illustrated Fig. 13. This plasmid is designated pGX-HIV/VN.

To be concrete, the third SIV immunogenic plasmid pGX10-HIV/VN comprises a gene (VN) formed by binding the HIV-1 vif and nef. The nef gene is modified by the deletion of codons for Arg137 and Arg138 which are known to play an important role in the downregulation activity of CD4 (J. Biol. Chem. 270;15307, 1995) so as to prevent the immunosuppressive effects of the Nef protein. Thus, this plasmid was devised to increase expression of fused Vif-Nef by fusing the signal sequence encoding 33 N-terminal amino acids of HSV gD to the 3' end of the VN gene so that the VN gene comes under direct transcription control of CMV promoter.

Meanwhile, it should be understood that various changes and modifications of methods for mutagenesis, types of promoter, and types and lengths of the glycoprotein signal sequence, other than those described above, can be made according to the

purpose for practicing the present invention, and such changes and modifications will be apparent to those skilled in the art.

(4) Forth SIV immunogenic plasmid

This novel plasmid is constructed by inserting a DNA sequence comprising any one of genes having from exon 1 to a full-length sequence of the HIV-1 tat gene, and a DNA sequence encoding signal peptide of secretory protein and the HIV-1 vpx gene fused to the 3' and 5' ends of the HIV-1 tat gene, respectively, into a vector, in which the genes and signal sequence are operably linked to the vector. The vector which can be used includes any mammalian cell expression vectors, preferably, a DNA vaccine vector optimized to induce immune response upon expression in muscle cells, DC, and T cells. More preferably, the vector is a vector including CMV promoter and optionally TPL sequence SV40 pA. Concrete examples of vectors which can be used in the present invention include, but are not limited to, pGX10 and pTV2; pVX1, pRC/CMV, pREP4 and pREP10 (though including RSV promoter), pcDNA1, pcDNA1.1, pcDNA3, pcDNA3/CAT, pRC/CMV and pRC/CMV2 supplied by Invitrogen Corp.; pCMV-script supplied by Stratagene; and pSI, pCI and pCI-neo supplied by Promega. The most preferred is pGX10.

The fourth plasmid thus constructed is delivered along with the first HIV immunogenic plasmid pGX10-HIV/GE and the second HIV immunogenic plasmid (for example, pGX10-HIV/dpol) to enhance the protection induced by the first and second immunogenic plasmids.

Additionally, the fourth HIV immunogeinc plasmid is delivered along with the first HIV immunogenic plasmid pGX10-HIV/GE, the second HIV immunogenic

plasmid (for example, pGX10-HIV/dpol) and the third HIV immunogenic plasmid (for example, pGX10-HIV/VN) to enhance the protection induced by the first and second immunogenic plasmids.

The tat gene is preferably used in a modified form since it may bring about immune disturbance, though it can be used in its full-length form. A region in the tat gene which can be modified comprises the entire gene except exon 1. The exon 1 of the tat gene expresses the enzyme activity (immune disturbance) of the Tat but the effect is less than that of exon 1+exon 2. The immune epitope included in exon 2 of the tat gene cannot be used. Therefore, it is the most preferable to use only the exon 1 site of the tat gene.

To the 3' end of the HIV-1 tat regulatory gene, a DNA sequence encoding a signal peptide of secretory protein is fused. As a result of such fusion, the transcription of the tat gene is directly controlled by the CMV promoter, thereby increasing expression levels of the Tat and Vpx proteins. Preferably, the DNA sequence encoding a signal peptide of glycoprotein is used as the DNA sequence encoding a signal peptide of secretory protein. Examples of the glycoprotein include herpes simplex virus glycoprotein gD, varicella zoster virus (VZV) gB, human cytomegalovirus (HCMV) gH, gL, gO, vesicular stomatitis virus (VSV) G protein, rotavirus outer capsid glycoprotein, VP7 and the like.

In one embodiment, another immunogenic plasmid of 4.2 kb is constructed by inserting exon 1 of the HIV-1 tat gene, and a DNA sequence encoding a signal peptide of HSV gD and the HIV-1 vpx gene fused to the 3' and 5' ends of the HIV-1 tat gene, respectively, into the MCS (multi-cloning site) of the vector pGX10 according to the present invention, in which the genes and the signal sequences are operably linked to

the vector. This plasmid is used as a third or fourth HIV immunogenic plasmid in the DNA vaccine against AIDS human patients. The detailed procedure for constructing this plasmid is described in Example 11 and illustrated Fig. 14. This plasmid is designated pGX-HIV/TV.

To be concrete, the fourth HIV immunogenic plasmid pGX10-HIV/TV comprises a gene (TV) formed by fusing exon 1 of the SIVmac239 tat gene and vpx gene. Thus, this plasmid was formed by fusing the signal sequence encoding 33 N-terminal amino acids of HSV gD to the 5' end of the TV gene so that the gene TV comes under direct transcriptional control of CMV promoter.

Meanwhile, it should be understood that various changes and modifications of methods for mutagenesis, types of promoter, and types and lengths of the glycoprotein signal sequence, other than those described above, can be made according to the purpose for practicing the present invention, and such changes and modifications will be apparent to those skilled in the art.

(5) Immunogenic plasmid comprising the HIV-1 regulatory genes vif, nef, tat and vpx

In a preferred embodiment of the present invention, a plasmid is constructed by inserting (i) the HIV-1 vif gene, and a DNA sequence encoding a signal peptide of secretory protein and the HIV-1 nef gene fused to the 3' and 5' ends of the HIV-1 vif gene, respectively, and (ii) a DNA sequence comprising any one of genes having from exon 1 to a full-length sequence of the HIV-1 tat gene, and a DNA sequence encoding a signal peptide of secretory protein and the HIV-1 vpx gene fused to the 3' and 5' ends of the HIV-1 tat gene, respectively, into a vector, in which the genes and signal

sequences are operably linked to the vector. Concrete examples of the vector which can be used in the present invention include, but are not limited to, pGX10 and pTV2; pVX1, pRC/CMV, pREP4 and pREP10 (though including RSV promoter), pcDNA1, pcDNA1.1, pcDNA3, pcDNA3/CAT, pRC/CMV and pRC/CMV2 supplied by Invitrogen Corp.; pCMV-script supplied by Stratagene; and pSI, pCI and pCI-neo supplied by Promega. The most preferred is pGX10.

The plasmid thus constructed is delivered along with the first HIV immunogenic plasmid pGX10-HIV/GE and the second HIV immunogenic plasmid (for example, pGX10-HIV/dpol) to enhance the protection induced by the first and second immunogenic plasmids. Also, this plasmid can reduce the number of immunogenic plasmid which should be prepared for a DNA vaccine, thereby lowering the production cost.

In a preferred embodiment of the present invention, the HIV-1 vif regulatory gene and the HIV-1 nef regulatory gene, which is fused at the 5' end of the HIV-1 vif regulatory gene, may be modified to remove its immunosuppressive effects. The modification can be effected by various methods. For example, Ser114-Leu150 in the gene vif can be modified (Fields Virology Third edition, p1901, Lippincott-Raven Col, 1996) and Arg137, Arg138 and Gly2 (involved in myristylation) in the gene nef can be modified.

In another preferred embodiment of the present invention, the tat gene is preferably used in a modified form since it may bring about immune disturbance, though it can be used in its full-length form. A region in the tat gene which can be modified comprises the entire gene except exon 1. The exon 1 of the tat gene expresses the enzyme activity (immune disturbance) of the Tat but the effect is less

than that of exon 1+exon 2. The immune epitope included in exon 2 of the tat gene cannot be used. Therefore, it is the most preferable to use only exon 1 site of the tat gene. The nef and tat genes can be independently modified. For example, there can be a case where the nef gene is modified while the tat gene is not modified, and where the tat gene is modified while the nef gene is not modified, or where both the nef and tat genes are modified.

To each 3' end of the HIV-1 vif and tat genes, a DNA sequence encoding a signal peptide of secretory protein is fused. As a result of such fusion, the transcription of the vif and tat genes is directly controlled by each CMV promoter, thereby increasing expression levels of the Vif and Nef proteins. Preferably, the DNA sequence encoding a signal peptide of glycoprotein is used as a signal sequence of secretory protein. Examples of the glycoprotein include herpes simplex virus glycoprotein gD, varicella zoster virus (VZV) gB, human cytomegalovirus (HCMV) gH, gL, gO, vesicular stomatitis virus (VSV) G protein, rotavirus outer capsid glycoprotein, VP7 and the like.

In one embodiment, an immunogenic plasmid of 7.5 kb is constructed by inserting exon 1 of the HIV-1 tat gene, and a DNA sequence encoding signal peptide of HSV gD and the HIV-1 vpx gene fused to the 3' and 5' ends of the HIV-1 tat gene, respectively, into a third HIV immunogenic plasmid comprising the HIV-1 vif gene, and a DNA sequence encoding a signal peptide of HSV gD and the modified HIV-1 nef gene (having codons for Arg137 and Arg138 deleted) fused to the 3' and 5' ends of the HIV-1 vif gene, respectively, to be operably linked to MCS (multi-cloning site) of the vector pGX10 according to the invention, in which the genes and the signal sequences are operably linked to the third HIV immunogenic plasmid. This plasmid is used as

an additional HIV immunogenic plasmid in the DNA vaccine against AIDS human patients. The detailed procedure for constructing this plasmid is described in Example 12 and illustrated Fig. 15. This plasmid is designated pGX-SIV/VNTV.

In another preferred embodiment, an immunogenic plasmid of 7.5 kb is constructed by inserting the HIV-1 vif gene, and a DNA sequence encoding signal peptide of HSV gD and the modified HIV-1 nef gene fused to the 3' and 5' ends of the HIV-1 vif gene, respectively, into a fourth HIV immunogenic plasmid comprising exon 1 of the HIV-1 tat gene, and a DNA sequence encoding signal peptide of HSV gD and the HIV-1 vpx gene fused to the 3' and 5' ends of the HIV-1 tat gene, respectively, to be operably linked to MCS (multi-cloning site) of the vector pGX10 according to the present invention, in which the genes and the signal sequences are operably linked to the fourth HIV immunogenic plasmid. This plasmid is used as an additional HIV immunogenic plasmid in the DNA vaccine against AIDS human patients. The detailed procedure for constructing this plasmid is described in Example 13 and illustrated Fig. 16. This plasmid is designated pGX-SIV/TVVN.

Meanwhile, it should be understood that various changes and modifications of methods for mutagenesis, types of promoter, and types and lengths of the glycoprotein signal sequence, other than those described above, can be made according to the purpose for practicing the present invention, and such changes and modifications will be apparent to those skilled in the art.

DNA VACCINE

The experiments by the inventors revealed that combined administration of immunogenic plasmids pGX10-SIV/GE and pGX10-SIV/dpol or immunogenic

plasmids pGX10-SIV/GE, pGX10-SIV/dpol, pGX10-SIV/VN and pGX10-SIV/TV to rhesus monkey elicited higher inhibition of both the proliferation of SIVmac239 in rhesus monkey and the reduction in the number of CD4+ cells, a typical symptom of AIDS development, as compared to combined administration of immunogenic plasmids pTV-SIV/GE and pTV-SIV/dpol. Therefore, compositions containing immunogenic plasmids of the invention are useful as vaccines for prophylaxis of AIDS.

Immunotherapy is a method for inhibiting virus proliferation by enhancing immune response to virus, rather than a method for introducing chemical substances which can inhibit enzymes necessary for proliferation of virus such as reverse transcriptase and protease. DNA immunotherapy has been applied to HIV infected chimpanzees (Boyer J D., et al., AIDS 14:1515-22, 2000; and Boyer J D., et al., J. Infect. Dis. 176:1501-9, 1997) or HIV infected humans (MacGregor RR J. Infect. Dis. 178:92-100, 1998). Since the therapy efficacy of vaccine correlates with its ability to elicit immune response to inhibit proliferation of virus, it is accepted by those in the art that vaccines showing good protection efficacy can be used in therapy. Therefore, compositions containing immunogenic plasmids of the invention can be used as vaccines for treatment of AIDS.

The administration manner and formulation of DNA vaccines of the invention for AIDS protection are in accordance with practices used for common vaccines, especially DNA vaccines for protection. DNA vaccines for AIDS therapy can be administered and formulated the same as DNA vaccines for AIDS protection in that they are used to increase immune response to AIDS virus.

DNA vaccine of the invention will preferably be administered by direct (in vivo) gene transfer. Naked DNA can be given by intramuscular, subcutaneous,

intravenous, intraarterial or buccal injection. Plasmid DNA may be coated onto gold particles and introduced biolistically with a "gene-gun" into the epidermis of the skin or the oral or vaginal mucosae (Fynan et al., Proc. Natl. Acad. Sci. USA 90:11478, 1993; Tang et al., Nature 356:152, 1992; Fuller et al., J. Med. Primatol. 25:236, 1996; Keller et al., Cancer Gene Ther., 3:186, 1996). DNA vaccine vectors may also be used in conjunction with various delivery systems. Liposomes have been used to deliver DNA vaccines by intramuscular injection (Gregoriadis et al, FEBS Lett.402:107, 1997) or into the respiratory system by non-invasive means such as intranasal inhalation (Fynan et al., supra). Other potential delivery systems include microencapsulation (Jones et al., 1998; Mathiowitz et al., 1997) or cochleates (Mannino et al., 1995, Lipid matrix-based vaccines for mucosal and systemic immunization. Vaccine Designs: The Subunit and Adjuvant Approach, M. F. Powell and M. J. Newman, eds., Pleum Press, New York, 363-387), which can be used for parenteral, intranasal (e.g., nasal spray) or oral (e.g., liquid, gelatin capsule, solid in food) delivery. DNA vaccines can also be injected directly into tumors or directly into lymphoid tissues (e.g., Peyer's patches in the gut wall). It is also possible to formulate the vector to target delivery to certain cell types, for example, to APC. Targeting to APC such as dendritic cells is possible through attachment of a mannose moiety (dendritic cells have a high density of mannose receptors) or a ligand for one of the other receptors found preferentially on APC. There is no limitation as to the route by which the DNA vaccine is delivered, nor the manner in which it is formulated, as long as the cells that are transfected can express antigen in such a way that an immune response is induced.

Where DNA vaccine of the present invention is administered to mucosa, it may be placed into a pharmaceutically acceptable suspension, solution or emulsion for

administration to mucosa. Suitable mediums include saline and liposomal preparations. More specifically, pharmaceutically acceptable carriers preferred for use with the gene expression plasmids of the invention may include sterile aqueous or non-aqueous solutions, suspensions, and emulsions suitable for ingestion, inhalation, or administration as a suppository to the rectum or vagina. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and certain organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Preservatives and other additives may also be present such as, for example, antimicrobials, antioxidants, chelating agents, and inert gases and the like. One skilled in the art will select among these available compounds depending upon the particular mucosal inductor site targeted, i.e., whether for ingestion or inhalation. Further, a composition of antigen-encoding polynucleotide preparations comprising gene expression plasmids may be lyophilized using means well known in the art, for administration by inhalation as an aerosol or subsequent reconstitution and use according to the invention.

Isotonic buffered solution is the preferred medium for maximal uptake of the gene plasmids contained in DNA vaccines of the invention. Further, use of absorption promoters, detergents, and mild chemical irritants is also preferred to enhance transmission of antigen-encoding polynucleotide preparation compositions through the point of entry and into contact with tissue adjacent to or containing a mucosal inductor site. For reference concerning general principles regarding promoters and detergents which have been used with success in mucosal delivery of organic and peptide-based drugs, see Chien, Novel Drug Delivery Systems, Ch. 4 (Marcel Dekker, 1992). Specific

information concerning known means and principles of nasal drug delivery are discussed in Chien, supra at Ch 5. Examples of suitable nasal absorption promoters are set forth at Ch. 5, Tables 2 and 3; milder agents are preferred. Suitable agents for use in the method of this invention for mucosal/nasal delivery are also described in Chang, et al., Nasal Drug Delivery, "Treatise on Controlled Drug Delivery", Ch. 9 and Table 3-4B thereof, (Marcel Dekker, 1992). Suitable agents which are known to enhance absorption of drugs through skin are described in Sloan, Use of Solubility Parameters from Regular Solution Theory to Describe Partitioning-Driven Processes, Ch. 5, "Prodrugs: Topical and Ocular Drug Delivery" (Marcel Dekker, 1992), and at places elsewhere in the text. "Detergents/Absorption Promoters" refers to chemical agents which are presently known in the art to facilitate absorption and transfection of certain small molecules, as well as peptides. "Mucosa" refers to mucosal tissues of a host wherever they may be located in the body including, but not limited to, respiratory passages (including bronchial passages, lung epithelia and nasal epithelia), genital passages (including vaginal, penile and anal mucosa), urinary passages (e.g., urethra, bladder), the mouth, eyes and vocal cords. "Point of Entry" refers to the site of introduction of the polynucleotide into a host, including immediately adjacent tissue. "Mucosal Inductor Site" refers to a site on the mucosa where uptake of the antigen-encoding polynucleotide preparation is sought, including, but not limited to, Waldeyer's ring, Peyer's patches, gut-associated lympohoid tissues, bronchial-associated lymphoid tissues, nasal-associated lymphoid tissues, genital-associated lymphoid tissues, and tonsils.

The dosage of DNA vaccine according to the present invention can be varied depending on administration manner, tissues to which DNA vaccine is administered,

such as skeletal muscle and skin, desired antibody titer, particular treatment requirement for immunization subject, etc. The effective amount of each plasmid contained in DNA vaccine of the present invention is from 0.01 to 0.2 mg/kg of weight, preferably 0.01 to 0.1 mg/kg of weight. The use of coated projectiles enables a smaller amount of the vaccine to be administered.

Lyophilized DNA Vaccine Formulations

The DNA vaccine composition can be lyophilized to increase its stability at room temperature, to reduce the requirement for costly cold storage, and to extend product shelf-life. The lyophilization process consists of three successive steps of freezing, primary drying and secondary drying. After freezing the product, the primary drying step involves lowering pressure and supplying heat for water vapor sublimation. During the secondary drying step, the residual absorbed moisture evaporates from the dried material.

In one embodiment, DNA vaccine of the present invention can be lyophilized as follows: (1) Determine the collapse temperature of the formulation by using freeze-drying microscopic analysis; (2) Place the vials on the freeze-drier shelves at room temperature and subsequently equilibrate at −1C for about 30 minutes; (3) Cool the shelves to −55C and hold that temperature for 2 hours; (4) Carry out primary drying at a product temperature of about −32C or 5C below the collapse temperature; (5) Carry out secondary drying at 35C (Complete the drying after adjusting the chamber pressure to between 55-120 mmHg); (6) Insert the stoppers into the vials under vacuum in the freeze-dryer (Crimp-seal the freeze-dried vials and store them at 2-8C).

A variety of excipients and lyoprotectants can be used in lyophilized DNA vaccine

formulations. The excipients include but are not limited to buffer of 0.9% NaCl + 10 mM sodium phosphate, pH 7.0 or 10 mM sodium citrate, pH 7.0. The lyoprotectants serve to protect biological molecules from freezing and drying processes and give mechanical support to the finished product. Examples of the lyoprotectant are PBS(phosphate-buffered saline, pH 7.0), PBS/4%, 12% or 15% trehalose, PBS/12% or 20% mannitol, PBS/15% or 20% lactose, PBS/4% sucrose, PBS/2% sorbitol, PBS/2% PEG(polyethylene glycol), PBS/4% trehalose/1% PEG/1% PVP(polyvinyl pyrolidone), PBS/4% mannitol/1% PEG/1% PVP, PBS/4% lactose/2% PEG and PBS/12% lactose/0.9% benzoyl alcohol.

ADJUVANTS

DNA vaccine of the invention can further contain adjuvant plasmids expressing immunoregulatory molecules such as cytokine proteins. Such adjuvant plasmids include but are not limited to plasmids expressing IL-1, IL-2, IL-4, IL-7, IL-12, IFN-γ, and GM-CSF. Examples of IL-4 expressing plasmid are plasmid pCAGGSIL-12(Fig. 22) and plasmid pGX10-hIL-12m(Fig. 17). The preferred adjuvant plasmid used in DNA vaccine of the invention is plasmid pGX10-hIL-12m.

Example

Restriction enzyme treatment: In the following Examples, the restriction enzyme treatment was performed as follows. 2 μg (1 μg/μℓ) of plasmid DNA or purified PCR product was mixed with 20 unit (2 μℓ) of a restriction enzyme (the restriction enzymes used in the following examples were products of Takara Shuzo Co.,

Ltd. and New England Biolab, Inc.) along with buffer solution (10-times concentrated solution) supplied by manufacturer. Distilled water was added to 50 $\mu\ell$. The reaction was performed at 37℃ for 2hr.

DNA segment ligation and transformation of E. coli: DNA solution treated with restriction enzyme was subjected to electrophoresis over 0.8% agarose gel (GIBCO-BRL). An agarose gel was cut to separate gel slice containing DNA segments of a desired size. The DNA segments were extracted and purified by means of a gel extraction kit (QIAEN). The DNA segments were added to a buffer solution with T4 DNA ligase (Takara) and were ligated at 16℃ for 10hr. E. coli was transformed by the ligated DNA segments according to the method described in Sambrook, et al., Molecular Cloning (2nd ed.), Chapter 1.74.

Identification of DNA having ligated plasmid in the transformed E. coli: A small amount of DNA was purified from the transformed E. coli according to the method described in Sambrook, et al., Molecular Cloning (2nd ed.), Chapter 1.25. The DNA was cut with restriction enzymes. Sizes of the resulting segments were measured and examine whether the plasmid contained the desired ligated fragment, based on the restriction enzyme map of the resultant DNA.

Purification of plasmid DNA: Pure DNA was obtained in a large amount according to the method described in Sambrook, et al., Molecular Cloning (2nd ed.), Chapter 1.3-1.4.

PCR amplification: PCR amplification was performed as follows. 200 pmol of two types of oligonucleotides (primers) were mixed with 20 ng of a template DNA, 10 unit of Takara exTaq (polymerase), 5 $\mu\ell$ of Takara exTaq 10x buffer solution, 5 $\mu\ell$ of 2.5mM dNTP mixture and distilled water to make a final volume of 50 $\mu\ell$. Then, the mixture was subjected to predenaturation for 4 min at 94℃, denaturation for 1 min at 94℃, annealing for 1 min at 52℃ and polymerization at 72℃ for 1 min per 1 kb of amplification product (for example, 0.5 min for 0.5 kb amplification; 3 min for 3 kb amplification) in order. This procedure was repeated for 30 cycles. For the final extension, the reaction was kept at 72℃ for 5 min. The used PCR apparatus was Perkin Elmer's GeneAmp PCR System 2400. The resulting PCR product was analyzed by electrophoresis over agarose gel and purified with a gel extraction kit of QIAGEN. The product was then cut with restriction enzymes and used in cloning for ligation with other DNA segments or other PCR products.

Other unspecified matters regarding subclonings were done by the methods described in Sambrook, et al., Molecular Cloning (2nd ed.), with minimal modification.

Example 1

Preparation of DNA vaccine vector pGX10 (Fig. 1)

A. Construction of vector pTV-3 (Fig. 1a)

2 μg of vector pMT-3 (Sambrook, et al., Molecular cloning 2nd Ed., vol. 3, 16.20; Kaufman RJ, et al., *Mol. Cell Biol.* 9,946-958, (1989)) was cut with HpaI (20 unit) and NheI (20 unit) according to the above-described restriction enzyme treatment. The resulting segment was mixed with dNTP (Takara) along with Klenow fragment

(New England Biolabs) (5 unit) to a final concentration of 100μM and let stand at 25℃ for 30min (blunt-end). After agarose gel electrophoresis, segment of 0.7 kb (including entire VAI and a part of SV40 polyA) was inserted into a unique Hpa I site of SV40 polyA part of vector pTV-2 (Lee, et al., *J. Virol.*, 72,8430-36, (1998)) to form vector pTV-3 of 5.3 kb.

B. Construction of vector pGX-1 (Fig. 1a)

PCR was conducted using the prepared vector pTV-3 as a template and the following primers.

CMV5 (TCG CGA CCC GGG CGA CGG CCA GTG AAT TGT ACC G): SEQ. ID. NO.: 1 (sense)

VA3 (TCG CGA GGC GCG CCA CGA GCC GCC GCG CCT GGA AGG): SEQ. ID. NO.: 2 (antisense)

The PCR product (2.0 kb) was cut with NruI according to the above-described restriction enzyme treatment.

Also, PCR was conducted using vector pZero-2 (Invitrogen) as a template and the following primers.

Zero5 (AAT ATT GTC GAC TTC AGA AGA ACT CGT CAA GAA G): SEQ. ID. NO.: 3 (sense)

Zero3 (AAT ATT GGG CCC GAA CAT GTG AGC AAA AGG CCA G: SEQ. ID. NO.: 4 (antisense).

The amplification product was cut with SspI, and the resulting DNA segment (1.8 kb) was joined with the above segment (2.0 kb) to form vector pGX-1 of 3.8 kb.

C. Construction of vector pGX10 (Fig. 1b)

Vector pGX-1 was cut with restriction enzymes XbaI and SalI. The larger DNA segment (3.1 kb) was separated according to the above-described procedure. Vector pGL3-Enhancer (Promega) was cut with restriction enzymes XbaI and SalI. The smaller DNA segment (0.5 kb) was separated by the same method as above. The separated segments were joined together to form vector pGX10.

Fig. 2 and Fig. 3 show the gene map of the prepared vector pGX10 (3.5 kb) and sequential position of all 3641 nucleotides, respectively.

Example 2

Preparation of plasmid pGX10-SIV/GE used as an immunogen (Fig. 5)

A. Construction of vector pTV-SIV/GE (Fig. 4a and Fig. 5b)

(a) The following oligonucleotides 1193Kpn and 3464Xba, which contain KpnI and XbaI sequences, respectively, were prepared:

1193Kpn (CGGGTCGGTACCAGACGGCG): SEQ. ID. NO.: 5 (sense)

3464Xba (ATCTAGAGGTATGGAGAAATAT): SEQ. ID. NO.: 6 (antisense).

Using these oligonucleotides as PCR primers and SIVmac239 DNA clone (GeneBank Accession No. M33262; Regier, et al., AIDS Res. Hum. Retroviruses, 6,1221-1231, (1990)) as a template, PCR amplification was conducted. The amplification product was cut with KpnI and XbaI. Nucleotide sequences 1193-3464 of SIVmac239 DNA, which numbers were designated based on Gene Bank numbering, starting from the primer end cut by the restriction enzyme, were inserted into pBluescript SK+ (Stratagene) which had been cut with SalI and XbaI, and subjected to

Klenow fragment treatment as in A of Example 1, to form vector pSK-SIVgag (5.3 kb) (Fig. 5a).

(b) The following oligonucleotides 6695Xba and 9641NotI, which contain XbaI and NotI sequences, respectively, were prepared:

6695Xba (GCCCTCTAGA AGCATGCTAT): SEQ. ID. NO.: 7 (sense)

9641NotI (GGAAGCGGCC GCCTCACTGA TACCCCTACC AA): SEQ. ID. NO.: 8 (antisense).

Using these oligonucleotides as PCR primers, and SIVmac239 DNA clone (GeneBank Accession: M33262) as a template, the amplified product was cut with restriction enzymes NotI and XbaI. The resulting segment was inserted into vector pSK-SIVgag, which had been partially cut with restriction enzymes NotI and XbaI, to form vector pSK-SIV/ge-1 (8.2 kb).

(c) The following oligonucleotides 8328Cla and 9535Xho were prepared:

8328Cla (ACTGTATCGATTGGAATTGG): SEQ. ID. NO.: 9 (sense)

9535Xho (CTCCCTCGAGTATTCATATACTGTCCCTGA): SEQ. ID. NO.: 10 (antisense).

Using the prepared oligonucleotides as primers and SIVmac239 clone DNA (GeneBank Accession: M33262) as a template, PCR amplification was conducted. The amplification product was inserted at SmaI site of pBluescript SK+ (Stratagene) to form vector pSK-SIVenv3 (4.2 kb).

(d) Vector pSK-SIV/ge-1 was cut with restriction enzymes ClaI and NotI. The resulting segment including the vector (6.7 kb) was joined with the segment not including the vector (1.2 kb), prepared by cutting pSK-SIVenv3 with restriction enzymes ClaI and NotI, to form vector pSK-SIV/ge (7.9 kb). The prepared pSK-

SIV/ge DNA was cut with KpnI and NotI and inserted at KpnI/NotI site of vector pTV2 (Lee SW, et al., *J. Virol.*, 72:8430-36, 1998) to form plasmid pTV-SIV/GE (10.0 kb).

E. coli DH5a transformed by the prepared plasmid pTV-SIV/GE according to the present invention was deposited at KCTC (Korean Collection for Type Cultures) of KRIBB (Korea Research Institute of Bioscience and Biotechnology) on November 27, 1999 (KCTC No. 0702BP).

B. Construction of plasmid pGX10-SIV/GE (Fig. 5c)

Plasmid pTV-SIV/GE was cut with restriction enzymes MluI and XhoI to prepare a segment (6.1 kb) including CMV promoter, TLP sequence and SIV gene. Also, pGX10 vector was cut with restriction enzymes MluI and XhoI to prepare a DNA segment (2.6 kb) not including CMV promoter or TLP sequence. Two DNA segments were joined together to form plasmid pGX10-SIV/GE (8.7 kb).

Example 3

Preparation of plasmid pGX10-SIV/dpol used as an immunogen (Fig. 6)

A. Construction of vector pTV-SIV/pol (Fig. 6)

(a) The following oligonucleotides having BamHI and XhoI sites were prepared:

BamHI (AATGGATCCA TAGCTAAGT AGAG): SEQ. ID. NO.: 11 (sense)

XhoI (ATTTCTCGAG GCTATGCCAC CTCTC): SEQ. ID. NO.: 12 (antisense).

Using these oligonucleotides as PCR primers, nucleotide sequences 3105-5668 of SIVmac239 DNA clone (GeneBank Accession: M33262) were amplified and cut

with restriction enzymes BamHI and XhoI. The PCR-amplified BamHI/XhoI segment (2.6 kb) was inserted at pSK-gDs/E2t, in which E2 has been deleted using restriction enzymes BglI and XhoI (Lee, et al., *J. Virol.*, 72,8430-6, (1998)), to form plasmid pSK-gDsSIV/pol (5.7 kb).

(b) In integrase region of plasmid pSK-gDsSIV/pol, nucleotide sequences 5130-5132 were deleted and nucleotide sequences 5133-5135 were mutated to express Ser117 instead of Asn117. As a result, pSK-gDsSIV/polm (5.7 kb) was formed. PCR was conducted using pSK-gDsSIV/pol as a template and the following oligonucleotide primers in order to mutate the pol gene:

(AGTGGTGCTA ACTTTGCTTC GCAA): SEQ. ID. NO.: 13 (sense)

(TGTGTGTAGA TGTGTAATAG GCC): SEQ. ID. NO.: 14 (antisense).

The amplification product was mixed with ATP to a final concentration of 100 μM and 10 units of T4 polynucleotide kinase (New England BioLabs) and incubated at 37℃ for 1hr, and then self-ligated. Trnsformation of E.coli was performed by using the ligated DNA, and pSK-gDsSIV/polm (5.7 kb) was generated.

(c) Vector pSK-gDsSIV/polm was cut with restriction enzymes NotI and XhoI, and inserted at the corresponding restriction enzyme sites of pTV2 (Lee, et al., *J. Virol.*, 72:8430, 1998) to form plasmid pTV-SIV/dpol (7.6 kb).

E. coli DH5a transformed with the prepared plasmid pTV-SIV/dpol according to the present invention was deposited at KCTC of KRIBB on November 27, 1999 (KCTC No. 0703BP).

B. Construction of plasmid pGX10-SIV/dpol (Fig. 6)

Plasmid pTV-SIV/dpol was cut with restriction enzymes NotI and XhoI. The

resulting segment (2.7 kb) including SIV gene was inserted into vector pGX10 (3.6 kb), which had been cut with the same restriction enzymes, to form plasmid pGX10-SIV/dpol (6.3 kb).

Example 4

Preparation of plasmid pGX10-SIV/VN used as an immunogen (Fig. 7)

A. Construction of pGX10-gDs (Fig. 7a)

The following oligonucleotides were prepared:

PstIgDs (CAA CTGCAG ATG GGG GGG GCT GCC G): SEQ. ID. NO.: 15 (sense)

gDsAsclNotI (ATT GCG GCC GCA GGC GCG CCG ATC TGA GAG AGG CAT CC): SEQ. ID. NO.: 16 (antisense).

PCR was conducted using these c oligonucleotides as primers and the vector pGX10-SIV/pol as a template. The amplification product was cut with restriction enzymes PstI and NotI. The resulting segment (0.1 kb) was inserted into vector pBluescriptSK (Stratagene) (3.0 kb), which had been cut with the same restriction enzymes, to form a plasmid pSK-gDs (3.1 kb).

B. Construction of pSK-gDs/Vif (Fig. 7b)

The following oligonucleotides were prepared:

AscI5'vif (T ATG GCG CGC CTG GAG GAG GAA AAG AGG): SEQ. ID. NO.: 17 (sense)

vif3'XbaINotI (AAAGCGG CCGC AAT CT AGA TCATGCCAG

TATTCCCAAG): SEQ. ID. NO.: 18 (antisense).

PCR was conducted using these synthetic oligonucleotides as primers and SIVmac239 clone (Gene Bank Accession No. M33262) as a template. The amplification product was cut with restriction enzymes AscI and NotI, and the resulting segment (0.6 kb) was inserted into pSK-gDs (3.1 kb), which had been cut with the same restriction enzymes, to form a plasmid pSK-gDs/Vif (3.7 kb).

C. Construction of pSK-5nef (Fig. 7b)

The following oligonucleotides were prepared:

BH5'nef (TAC GGA TCC ATG GGT GGA GCT ATTT T): SEQ. ID. NO.: 19 (sense)

5'SpeI (TCT ACT AGT ACT GTA ATA AAT CCC TTC): SEQ. ID. NO.: 20 (antisense).

PCR was conducted using these synthetic oligonucleotides as primers and SIVmac239 clone (Gene Bank Accession No. M33262) as a template. The PCR product was cut with restriction enzymes BamHI and SpeI, and the resulting segment was inserted between BamHI and SpeI sites of pBluscript SK+ (Stratagen) to form a plasmid pSK-5nef (3.0 kb).

D. Construction of pSK-nefM (Fig. 7b)

The following oligonucleotides were prepared:

Spe5'nef (AGA ACT AGT AGA ATC TTA GAC ATA TA): SEQ. ID. NO.: 21 (sense)

3'nefNotI (AAA GCGGCCGC TGT TTC AGC GAG TTT): SEQ. ID. NO.: 22

(antisense).

PCR was conducted using these oligonucleotides as primers and SIVmac239 clone (Gene Bank Accession No. M33262) as a template. The PCR product was cut with restriction enzymes SpeI and NotI. The resulting segment (0.4 kb) was inserted into plasmid pSK-5nef (3.4 kb), which had been cut with restriction enzymes SpeI and NotI, to form a plasmid pSK-nefM (3.8 kb).

E. Construction of pSK-SIV/VN (Fig. 7b)

PCR was carried out using vector pSK-nefM as a template and the following oligonucleotides as primers:

Xba5'nef (ACC TCT AGA ATG GGT GGA GCT ATT T): SEQ. ID. NO.: 23 (sense)

3'nefNotI (AAA GCGGCCGC TGT TTC AGC GAG TTT): SEQ. ID. NO.: 22 (antisense).

The product was cut with restriction enzymes NotI and XbaI, and the resulting segment (0.8 kb) was inserted into pSK-gDs/Vif (3.7 kb), which had been cut with restriction enzymes NotI and XbaI, to form a plasmid pSK-VN (4.5 kb).

F. Construction of pGX10-SIV/VN (Fig. 7c)

Vector pSK-VN was cut with restriction enzymes AscI and NotI. The smaller DNA segment (1.4 kb) was ligated with DNA segment (3.7 kb) prepared by cutting pGX10-SIV/TV (This could be prepared by using pSK-gDs/Vif and pGX10 intermediates previously) with restriction enzymes AscI and Not I, to form a plasmid pGX10-SIV/VN (5.1 kb).

Example 5

Preparation of plasmid pGX10-SIV/TV used as an immunogen (Fig. 8)

A. Construction of pSK-gDs/tat (Fig. 8a)

PCR was conducted using SIVmac239 clone (Gene Bank Accession No. M33262) as a template and the following synthetic oligonucleotides as primers:

AscI5'tat (TAT GGCG CGCC TGG AGA CAC CCT TGA GG): SEQ. ID. NO.: 24 (sense)

tat3'NheNotI (AAA GCGG CCG CAA TCT AGA GTT TGA TGC AGA AGA TGT A): SEQ. ID. NO.: 25 (antisense).

The product was digested with restriction enzymes AscI and NotI to obtain Exon 1 (0.3 kb) of tat gene, which was then inserted into pSK-gDs/Vif (3.1 kb) to form a plasmid pSK-gDs/tat (3.4 kb).

B. Construction of pSK-SIV/TV (Fig. 8a)

PCR was conducted using SIVmac239 clone (Gene Bank Accession No. M33262) as a template and the following synthetic oligonucleotides as primers.

Xba5'vpx (AAA TCTAGA ATG TCA GAT CCC AGG GAG): SEQ. ID. NO.: 26 (sense)

3'vpxNoI (AAAA GCGG CCGC TTA TGC TAG TCC TGG AGG): SEQ. ID. NO.: 27 (antisense).

The product was cut with restriction enzymes NotI and XbaI, and the resulting DNA segment of 0.3 kb was inserted into pSK-gDs/tat, which had been cut with

restriction enzymes NotI and NheI, to form pSK-SIV/TV (3.7 kb).

C. Construction of plasmid pGX10-SIV/TV (Fig. 8b)

Plasmid pSK-SIV/TV was cut with restriction enzymes PstI and NotI. The smaller DNA segment (0.7 kb) was inserted into pGX10, which had been cut with the same restriction enzymes, to form pGX10-SIV/TV (4.3 kb).

Example 6

Construction of pGX10-SIV/VNTV (Fig. 9)

The following oligonucleotides were prepared:

Sal5CMV (CCCG GTCGAC GGCCAG TGA ATT G): SEQ. ID. NO.: 28 (sense)

Sal3enh (CTT CTG AA GTCGAC GGA TCC GC): SEQ. ID. NO.: 29 (antisense).

PCR was conducted using these oligonucleotides as primers and plasmid pGX10-SIV/TV as a template. The product was cut with restriction enzyme SalI, and the resulting segment 2.4 kb was inserted into pGX10-SIV/VN, which had been cut with the same restriction enzyme, to form a plasmid pGX10-SIV/VNTV.

Example 7

Construction of pGX10-SIV/TVVN (Fig. 10)

PCR was conducted using plasmid pGX10-SIV/VN as a template and the same

oligonucleotides used in Example 6, i.e., Sal5CMV (sense) and Sal3enh (antisense), as primers. The product was cut with restriction enzyme SalI, and the resulting segment of 3.2 kb was inserted into pGX10-SIV/TV, which had been cut with the same restriction enzyme, to form a plasmid pGX10-SIV/TVVN.

The following Examples illustrate the preparation of HIV vaccine immunogenic plasmids by substituting SIVmac239 genes with corresponding HIV-1 genes. HXB2 and JRCSF were used as HIV-1 gene.

Example 8

Preparation of plasmid pGX10-HIV/GE used as an immunogen (Fig. 11)

Plasmid pTX GE(Lee A H et al., Vaccine 17:473-9, 1999) includes gag, env and rev genes of HXB2 (Gene Bank Accession No. K03455) as HIV gene and does not express and tat genes. Plasmid pGX10-HIV/GE (9.7kb) was constructed by ligating DNA fragment of 7.5kb (including CMV promoter, TPL sequence, HIV genes and portion of SV40 polyA), which had been obtained by digesting plasmid pTX GE with restriction enzymes HpaI and MluI, with DNA fragment of 2.2kb (including replication orgin and kanamycine resistance gene), which had been obtained by digesting vector pGX10 with restriction enzymes HpaI and MluI.

Example 9

Preparation of plasmid pGX10-HIV/dpol used as an immunogen (Fig. 12)

A. Construction of pSK-polMjr (Fig. 12a)

(a) The following oligonucleotides were synthesized:

BH5pol (AAT GGA TCC ATT AGT CCT ATT GA): SEQ. ID. NO.: 30 (sense)

PstIN (GCC CTG CAG TGT ATG TAT TGT TGT TA): SEQ. ID. NO.: 31 (antisense).

PCR amplification was carried out using these synthetic oligonucleotides as PCR primers, and proviral genes (pYK-JRCSF, NIH, AIDS Research and Reference Reagent Program) of HIV-1 JR-CSF (Gene Bank Accession No. M38429) as templates for PCR. The product obtained thereby was digested with restriction enzymes BamHI and PstI to produce DNA fragment of 2.0kb. The resulting DNA fragment was inserted into pBluescriptSK+ (Stratagen), which had been cut with the same restriction enzymes BamHI and PstI, to form plasmid pSK-5pol (5.0kb).

(b) The following oligonucleotides were synthesized:

Pst3pol (ACA CTG CAG GGC AGC AAT TTC ACC): SEQ. ID. NO.: 32 (sense)

polClaI (CTT ATC GAT GTT CTA ATC CTC ATC): SEQ. ID. NO.: 33 (antisense).

PCT amplification was carried out using these synthetic oligonucleotides as PCR primers, and HIV-1 JR-CSF clones as templates for PCR. The product obtained thereby was digested with restriction enzymes PstI and ClaI. The resulting DNA fragment

was inserted into plasmid pSK-5pol, which had been cut with the same restriction enzymes PstI and ClaI, to form plasmid pSK-polMjr (5.5kb).

(c) Plasmid pSK-polMjr was digested with restriction enzymes BamHI and NaeI to obtain DNA fragment of 2.8kb including HIV-1 pol gene. Plasmid pSK-gDs/E2t (Lee et al., J. Virol., 72:8430, 1998) was digested with the same restriction enzymes BamHI and NaeI to obtain DNA fragment of 2.8kb. The two DNA fragments were ligated to form plasmid pSK-gDs/polMjr of 5.6kb.

B. Construction of pGX10-HIV/dpol (Fig. 12b)

Plasmid pSK-gDs/polMjr was digested with restriction enzymes NotI and XhoI to obtain DNA fragment of 2.6kb. The resulting DNA fragment was inserted into NotI and XhoI sites of vector pGX10 to form pGX10-HIV/dpol (6.2kb).

Example 10

Preparation of pGX10-HIV/VN (Fig. 13)

A. Construction of pSK-gDs/Vifjr (Fig. 13a)

The following oligonucleotides were synthesized:

AscIvif (ATC GGCGCGC CTG GAA AAC AGA TGG CAG GT): SEQ. ID. NO. 34 (sense)

VifXbaI (CAT TCT AGA GTG TCC ATT CAT TGT ATG GC): SEQ. ID. NO.

35 (antisense).

PCT amplification was carried out using these synthetic oligonucleotides as PCR primers, and HIV-1 JR-CSF clones as templates for PCR. The product obtained thereby was digested with restriction enzymes PstI and ClaI. The resulting DNA fragment was inserted into plasmid pSK-5pol, which had been cut with the same restriction enzymes PstI and ClaI, to form plasmid pSK-polMjr (5.5kb).

B. Construction of pSK-nefMjr (Fig. 13b)

(a) The following oligonucleotides were synthesized:

BH5'nef-HIV (TAC GGA TCC ATG GGT GGC AAG TGG TCA): SEQ. ID. NO. 25 (sense)

5'SpeI (ATC ACT AGT TGA GTA AAT TAG CCC TTC): SEQ. ID. NO. 36 (antisense).

PCT amplification was carried out using these synthetic oligonucleotides as PCR primers, and HIV-1 JR-CSF clones (GeneBank Accession No. M38429) as templates for PCR. The product obtained thereby was digested with restriction enzymes BamHI and SpeI. The resulting DNA fragment was inserted into plasmid pBluscriptSK+ (Stratagen), which had been cut with the same restriction enzymes BamHI and SpeI, to form plasmid pSK-5nefjr (3.3kb).

(b) The following oligonucleotides were synthesized:

Spe5'nef-HIV (TCA ACT AGT GAT ATC CTT GAT CTG TGG): SEQ. ID. NO. 37 (sense)

3'nefNotI-HIV (GAT GCGGCCGC TCA GCA GTC CTT GTA GTA): SEQ. ID. NO. 38 (antisense).

PCT amplification was carried out using these synthetic oligonucleotides as PCR primers, and HIV-1 JR-CSF clones (GeneBank Accession No. M38429) as templates for PCR. The product obtained thereby was digested with restriction enzymes SpeI and NotI. The resulting DNA fragment of 0.3 kb was inserted into plasmid pSK-5nefjr, which had been cut with the same restriction enzymes SpeI and NotI, to form plasmid pSK-5nefMjr (3.6kb).

C. Construction of pSK-VNjr (Fig. 13b)

PCT amplification was carried out using the following synthetic oligonucleotides as PCR primers and pSK-nefM as template:

Xba5'nef (CAC TCT AGA ATG GGT GGC AAG TGG TCA): SEQ. ID. NO.: 39 (sense)

3'nefNotI-HIV (GAT GCGGCCGC TCA GCA GTC CTT GTA GTA): SEQ. ID. NO.: 38(antisense).

The product was digested with restriction enzymes NotI and XbaI. The resulting DNA fragment of 0.6kb was inserted into pSK-gDs/vifjr, which had been cut with restriction enzymes XbaI and NotI, to form pSK-VNjr of 4.3kb.

D. Construction of pGX10-HIV/VN (Fig. 13C)

Vector pSK-VNjr was digested with restriction enzymes AscI and NotI. The resulting small DNA fragment of 1.2kb was inserted into pGX10-HIV/TV, which had been cut with restriction enzymes PstI and NotI, to form pGX10-HIV/VN of 4.9kb.

Example 11

Preparation of pGX10-HIV/TV (Fig. 14)

A. Construction of pSK-gDs/tatjr(Fig. 14a)

The following oligonucleotides were synthesized:

AscI5'tat-HIV (ATC GGCG CGCC TGG AGC CAG TAG ATC CT): SEQ. ID. NO. 40 (sense)

tat3'XbaI-HIV (CCC TCT AGA CTT TGG TAG AGA AAC TTG): SEQ. ID. NO. 41 (antisense).

PCT amplification was carried out using these synthetic oligonucleotides as PCR primers, and HIV-1 JR-CSF clones (GeneBank Accession No. M38429) as templates for PCR. The product obtained thereby was digested with restriction enzymes AscI and NotI. The resulting DNA fragment (exon 1 of tat gene, 0.2kb) was inserted into plasmid pSK-gDs/Vif, which had been cut with the same restriction enzymes AscI and NotI, to form plasmid pSK-gDs/tatjr (3.3kb).

B. Construction of pSK/TVjr (Fig. 14a)

The following oligonucleotides were synthesized:

Xba5'vpu (CCC TCT AGA ATG CAA CCT TTA CAA AT): SEQ. ID. NO. 42 (sense)

3'vpuNotI (CGA GCGGCCGC CTA CAG ATC ATT AAT GTC): SEQ. ID. NO. 43 (antisense).

PCT amplification was carried out using these synthetic oligonucleotides as PCR primers, and HIV-1 JR-CSF clones (GeneBank Accession No. M38429) as templates for PCR. The product obtained thereby was digested with restriction enzymes NotI and XbaI. The resulting DNA fragment of 0.2kb was inserted into plasmid pSK-gDs/tatjr, which had been cut with the same restriction enzymes NotI and XbaI, to form plasmid pSK-TVjr (3.5kb).

C. Construction of pGX10-HIV/TV (Fig. 14b)

Vector pSK-TVjr was digested with restriction enzymes AscI and NotI. The resulting small DNA fragment of 0.5kb was inserted into pGX10-HIV/TV of 3.7kb, which had been cut with restriction enzymes AscI and NotI, to form pGX10-HIV/TV of 4.2kb.

Example 12

Preparation of pGX10-HIV/VNTV (Fig. 15)

The following oligonucleotides were synthesized:

Sal5CMV (CCCG GTCGAC GGCCAG TGA ATT G): SEQ. ID. NO. 28 (sense)

Sal3enh (CTT CTG AA GTCGAC GGA TCC GC): SEQ. ID. NO. 29 (antisense).

PCT amplification was carried out using these synthetic oligonucleotides as PCR primers, and pGX10-HIV/TV as templates for PCR. The product obtained thereby was digested with restriction enzyme SalI. The resulting DNA fragment of 2.3kb was inserted into plasmid pGX10-HIV/VN, which had been cut with the same restriction enzyme SalI, to form plasmid pGX10-HIV/VNTV.

Example 13

Preparation of pGX10-HIV/TVVN (Fig. 16)

PCT amplification was carried out using the following synthetic oligonucleotides as PCR primers, and pGX10-HIV/VN as template for PCR:

Sal5CMV (CCCG GTCGAC GGCCAG TGA ATT G): SEQ. ID. NO. 28 (sense)

Sal3enh (CTT CTG AA GTCGAC GGA TCC GC): SEQ. ID. NO. 29 (antisense).

The product obtained thereby was digested with restriction enzyme SalI. The resulting DNA fragment of 3.0kb was inserted into plasmid pGX10-HIV/TV, which had been cut with the same restriction enzyme SalI, to form plasmid pGX10-HIV/TVVN.

Example 14

Construction of pGX10-hIL-12m (pGX10-hp35/IRES/hp40) (Fig. 17)

A. Construction of pSK-hp35 and pSK-hp40 (Fig. 17a)

Using RT-PCR (PCR System 2400, Perkin Elmer) with complementary primers from NC37 cell, human B cell activated with PMA (phorbol myrystic acetate), cDNAs encoding human p35 subunit of 820 bp and human p40 subunit of 1,050 bp were cloned and amplified. As the complementary primers for amplification of human p35 subunit, the following oligonucleotides were used:

hp35 (5'-CCCGGGAAAGTCCTGCCGCGCCTCG-3'): SEQ. ID. NO. 44 (sense)

hp35 (5'-ACAACGGTTTGGAGGGA-3'): SEQ. ID. NO. 45 (antisense).

And, As the complementary primers for amplification of human p35 subunit, the following oligonucleotides were used:

hp40 5'-AGAGCACCATGGGTCACCAGCAGTTGG-3': SEQ. ID. NO. 46 (sense)

hp40 5'-CGATGCGGCCGCACCTAACTGCAGGG-3': SEQ. ID. NO. 47

(antisense).

Each amplified cDNA was subcloned into starting vector pBluescriptSK+ (Stratagene). Genes encoding P35 and p40 subunits was inserted into SmaI site of vector pBluescriptSK+ to pSK-hp35 of 3.8kb and pSK-hp40 of 4.0kb, respectively.

B. Construction of pSK-IRES (Fig. 17a)

A bicistronic vector co-expressing genes encoding p35 and p40 subunits was constructed. The IRES gene of EMCV was produced by RT-PCR and digested with restriction enzyme EcoRV. The resulting DNA fragment was inserted into EcoRV site of vector pBluescriptSK+ (Stratagene) to form vector pSK-IRES of 3.5kb. Then, the following oligonucleotides were used as complementary primers:

IRES (5'-AAGATATCGAATTCCCCCTC-3'): SEQ. ID. NO. 48 (sense)

IRES (5'-TTGCCATGGCCATATTTATCA-3'): SEQ. ID. NO. 49 (antisense).

C. pSK-hp35/IRES/hp40 (Fig. 17a)

Plasmid pSK-hp35 was digested with restriction enzymes SmaI and NotI and filled with T4 DNA polymerase to obtain hp35 fragment of 0.8kb. This DNA fragment was inserted into DNA fragment of 3.5kb formed by digesting vector pSK-IRES with restriction enzyme EcoRV to construct pSK-hp35/IRES of 4.3kb. The hp40 DNA fragment of 1.0kb obtained by digesting pSK-hp40 with restriction enzymes NcoI and NotI was inserted into pSK-hp35/IRES,

which had been cut with the same restriction enzymes, to construct the plasmid co-expressing genes encoding p35 and p40 subunits. The construct was digested with restriction enzymes SmaI and ClaI and ligated with ligase so that portion of restriction enzyme site upstream of hp35 was removed. The plasmid obtained thereby is designated pSK-hp35/IRES/hp40.

D. pSK-hp40-N222L (Fig. 17b)

PCR was carried out using T7 primer and hp40-N222L antisense primer and pSK-hp40 as template to substitute amino acid 222, asparagine, of hp40 with leucine. Similarly, second PCR was conducted using T3 primer and hp40-N222L sense primer. As results, two PCR fragments sharing common site including mutational point were formed. The second PCR was carried out using a mixture of those fragments as template and flanking primer to produce fusion product thereof. The resulting fusion DNA fragment was digested with restriction enzymes NcoI and NotI and inserted into vector pBluescriptSK+ of 3.0kb, which had been cut with restriction enzyme SmaI, to construct plasmid pSK-hp40-N222L of 4.0kb. Then, the following oligonucleotides were used as primer:

T7 (5'-GTAATACGACTCACTATAGGGC-3'): SEQ. ID. NO. 50 (sense)

hp40-N222L (5'-TATGAGCTCTACACCAGCAGC-3'): SEQ. ID. NO. 51 (antisense).

The bolded part corresponds to base sequence replaced for wild type hp40. The underlined part indicates SacI restriction site formed by mutation.

E. Construction of pSK-hp35/IRES/hp40-N222L (Fig. 17b)

Using restriction enzymes NcoI and NotI, hp40-N222L fragment of plasmid pSK-hp40-N222L was substituted with hp40 fragment of plasmid pSK-hp35/IRES/hp40 to construct plasmid pSK-hp35/IRES/hp40-N222L of 5.3kb.

F. Construction of pGX10-hp35/IRES/hp40-N222L (Fig. 17c)

To transfer fragment hp35/IRES/hp40-N222L into vector which can be expressed in mammalian cells, the following oligonucleotides were synthesized:

hIL-12 (5'-AACTCGAGGTCGACGGTATC-3'): SEQ. ID. NO. 54 (sense)

hIL-12 (5'-TTCTCGAGCGGCCGCACCT-3'): SEQ. ID. 55 (antisense).

PCR amplification was carried out using these synthetic oligonucleoties as primers and vector pSK-hp35/IRES/hp40-N222L as template to obtain fragment hp35/IRES/hp40-N222L. This fragment was digested with restriction enzyme XhoI and inserted into vector pGX10, which had been cut with restriction enzyme XhoI, to construct plasmid pGX10-hp35/IRES/hp40-N222L (or pGX10-hp35/IRES/hp40) of 5.9kb.

Experimental Example 1

Expression of vector pGX10

As described below, human growth hormone (hGH) gene was inserted into the

multi-cloning site of pGX10 vector according to the present invention, and its expression level was compared to that of control vector. The test result was shown in Fig. 18.

A. Preparation of plasmid that expresses hGH

Preparation of pTV2/hGH (Fig. 19): A PCR amplification was conducted using DNA having a human growth hormone gene (GeneBank Accession No. K02382; Ikehara M., et al., *Proc. Natl. Acad. Sci U.S.A.* 81,5956-60, (1984)) as a template and the following oligonucleotides as primers:

hGHF (AAGAA TTC GAT ATG TTCCCAA CTAT TC): SEQ. ID. NO.: 56 (sense)

hGHR (TTT TCT AGA ATTA GAAGCC ACAC GACC): SEQ. ID. NO.: 57 (antisense).

The amplification product was cut with restriction enzymes EcoRI and XbaI, and the resulting segment was inserted into pTV2, which had been digested with the same restriction enzymes, to form a plasmid pTV2/hGH.

Preparation of pGX1/hGH (Fig. 20): pTV2/hGH was cut with restriction enzymes EcoRI and XbaI. The resulting DNA segment of 0.6 kb was inserted into vector pGX1, which had been digested with the same restriction enzymes, to form a plasmid pGX1/hGH.

Preparation of pGX10/hGH (Fig. 24): pTV2/hGH was cut with restriction enzymes EcoRI and XbaI. The resulting DNA segment of 0.6 kb was inserted into vector pGX10, which had been cut with restriction enzymes EcoRI and XbaI, to form a plasmid pGX/hGH.

Preparation of pGX0/hGH (Fig. 23): PCR amplification was carried out using

pZero-2 (Invitrogen) as a template and the following oligonucleotides as primers:

KanXmF (TTG GAA AAC GTT CTT CGG GCG GCC TAT TGG TTA AAA AAT GAG C): SEQ. ID. NO.: 58 (sense)

KanXmR (CTT GAA CGT TTT CCT TTT CAC GTA GAA AGC): SEQ. ID. NO.: 59 (antisense).

The amplification product was cut with restriction enzyme XmnI, and inserted into vector pTV2, which had been cut with the same restriction enzyme XmnI, to form vector pGX0 (Fig. 23a):

pGX0/hGH was prepared by digesting pTV2/hGH with restriction enzymes EcoRI and XbaI and inserting the resulting DNA segment of 0.6 kb into vector pGX0, which had been cut with the same restriction enzymes EcoRI and XbaI (Fig. 23b).

B. Transfection of HeLa cells

(a) 2.0×10^5 cells were placed on each well of a 6-well plate. After adding 2 ㎖ of DMEM (10% FCS) culture medium, it was cultured for 18 hours at 37℃ and under 5% CO_2. (b) 8 ㎍ of hGH-expressing plasmid DNA was mixed and stirred with 100 ㎕ of 0.2M $CaCl_2$ solution. (c) The solution of (b) was added dropwise to 100 ㎕ of 2X HBS solution and let stand for 15 minutes at room temperature. (d) The solution of (c) was mixed gently and dispensed on cells cultured in (a) using a pipette. (e) After culturing for 12 hours under the same condition as (a), the culture medium was changed freshly and culturing was continued for additional 24 hours. Then, hGH in the culture medium was quantified.

C. hGH quantification

Roche's hGH ELISA kit (Product No. 1585878) was used to determine an amount of hGH in the culture medium of transfected cells. The culture medium was

diluted 200 times with the provided sample buffer solution, and other details followed the manufacture's instructions.

The values in Fig. 18 represent relative expression levels (hGH concentration in culture medium) of each vector, when the hGH expression of pTV2/hGH was defined as 100. That is, the larger the value, the higher the hGH levels in the culture medium expressed after transfection of vector DNA. Therefore, it can be seen from Fig. 18 that vector pGX10 of the present invention expresses three times more hGH than control group vector pTV2.

Experimental Example 2

Immune induction of vector pGX10

hGH-expressing plasmid DNAs (pTV2/hGH, pGX0/hGH and pGX10/hGH) prepared and purified in Experimental Example 1 were separately dissolved in a physiological saline to the concentration of 1 $\mu g/\mu \ell$. 100 $\mu \ell$ of this solution was injected in leg muscle (tibialis anterior muscle) of 8 weeks old female BALB/c mouse, and its immune response was observed. Three weeks after injection, blood was drawn from subocular vein of the mouse and analyzed by antibody ELISA. The antibody ELISA was carried out according to the method of Song, et al. in *J. Virol.*, 74, 2920, 2000. After 9 weeks, 100 μg of DNA solution was injected by the same method. After 11 weeks, blood was drawn from the mouse by the same method, and the relative IgG amount was measured by absorption at 450nm. The result was shown in Fig. 25.

In Fig. 25, amounts of anti-hGH antibody before immunization were spotted on a left side as O.D (absorption) at 405 nm. The central spots indicate the O.D values of

anti-hGH in blood serum collected at 3 weeks after vaccination. Spots on the right side are the O.D values of anti-hGH in blood serum collected at 9 weeks after vaccination. Higher O.D values mean stronger anti-hGH responses as induced. Therefore, it can be seen from Fig. 25 that immunization using pGX10-hGH of the present invention is superior to that of control group pTV-hGH.

Experimental Examples 1 and 2 demonstrate that vector pGX10 is superior to the control group vector pTV in terms of their in vitro expression levels and antibody production after immunization.

Experimental Example 3

Evaluation I on immune efficacy of plasmids according to the present invention

A. Immunization of monkey using immunogenic plasmids (see the test protocol shown in Fig. 26)

Plasmids pTV-SIV/GE and pTV-SIV/dpol were dissolved in saline (0.85% NaCl) to produce 1 ㎎/㎖ solutions as controls. For monkeys (rhesus macaque monkey) of Group 2 and Group 5, each group having 3 monkeys, 400 ㎍ of pTV-SIV/GE and 400 ㎍ of pTV-SIV/dpol (total 800 ㎍) were injected in the monkey's leg muscle at four sites.

Plasmids pGX10-SIV/GE, pGX10-SIV/dpol, pGX10-SIV/VN and pGX10-SIV/TV according to the present invention were separately dissolved in saline (0.85% NaCl) to form four solutions of 2 ㎎/㎖ concentration. For monkeys of Group 3 (3 monkeys), 400 ㎍ (2 ㎎/㎖) of each solution (total 1.6 ㎎) was injected in the

monkey's leg muscle at four sites.

B. Immunization of monkey using immunogenic and adjuvant plasmids (Cf.: Fig. 26-Test Protocol)

Plasmids pGX10-SIV/GE, pGX10-SIV/dpol, pGX10-SIV/VN and pGX10-SIV/TV and adjuvant plasmid pGX10-hIL-12m according to the present invention were separately dissolved in saline (0.85% NaCl) to form five solutions of 2.5 ㎎/㎖ concentration. For 3 monkeys of another test group (Group 4), 400 ㎍ (2.5 ㎎/㎖) of each solution (total 2 ㎎) was injected in the monkey's leg muscle at four sites.

After 8 weeks, 17 weeks, 25 weeks and 44 weeks from the first immunization, for every monkeys, the same DNA as one used at the first immunization was injected by the same method.

C. Determination of immune cells infected by SIVmac239 in blood

In order to identify to what extent the plasmid DNAs induce an immune response that inhibits proliferation of virus SIVmac239 in the monkey, SIVmac239 infection of PBMC (peripheral blood mononuclear cells) was examined. The infection was measured by counting infectious immune cells in the blood immune cells.

2 weeks after the final immunization (46 weeks after the first immunization), 10X of the predetermined MID50 (monkey infectious dose) of SIVmac239 (Deutsches Primatenzentrum, Germany; dosage: 1 ㎖) was injected by intravenous injection. 2 weeks, 4 weeks, 8 weeks, 12 weeks, 16 weeks, 20 weeks and 24 weeks after SIVmac239 infection, PBMCs from blood of the control group and the test group monkeys were separated, and the number of PBMCs infected by SIV in one million

cells are determined by limiting-dilution cocultivation (Lu, et. al., *J. Virol.* 70:3978, 1996). This method was as follows: 6 ㎖ of blood obtained from monkey (treated with citric acid) was put in a tube (lympho-prep, Greiner) containing 3 ㎖ of Ficoll-hypaque (Pharmacia). The blood was centrifuged for 20 min at 20℃ and 1200xg to separate red blood cells, PBMCs and plasma. Separated PBMCs were subjected to continuous dilution to reduce the number of cells by half from 1×10^6. Then, 10^5 C8166 target cells were added to the final dilution and cultured together (culture medium: RPMI1640 + 10% fetal bovine serum). After 3-4 days, half the culture medium including cells was removed and fresh culture medium was added. Repeating this procedures, the cultivation was carried out for 2 weeks. Upon completion of cultivation, the supernatant was taken (after centrifuging for 10min at 800xg). p27 antigen detection ELISA was carried out using a Coulter kit following the manufacturer's instruction. The final PBMC dilution that exhibits positive reaction was identified, and number of infectious cells per one million PBMCs was determined. If a dilution exhibits p27 positive reaction for 2.5×10^5 PBMCs and p27 negative reaction for the next dilution (1.25×10^5 PBMCs), the infectious cell ratio is 4/1,000,000.

The results are shown in Fig. 27. Reduction of infectious PBMCs means that cells wherein SIVmac239 was capable of replicating are reduced, which in turn means that SIVmac239 replication in the monkey body was controlled.

Fig. 27 shows number of infectious cells per one million PBMCs 0, 2, 4, 8, 12 and 16 weeks after SIVmac239 infection for each monkey group. For monkeys immunized with pTV-SIV/GE and pTV-SIV/dpol (Group 2+5), the number of infectious cells dropped to 0.13% (128/1000000) after 8 weeks, and 24 weeks after, for

two of the five monkeys, number of infectious cells was less than 1 (per 1,000,000), for one monkey, it dropped to 16; which was about 100-1000 times more efficient in inhibiting viral replication, compared to monkeys immunized with vector (Group 1; 1024-2048). For the other two of the five monkeys in Group 2+5, number of infectious cells after 24 weeks dropped to 128-256, which was only 8 times more efficient than Group 1. For three monkeys immunized with plasmids pGX10-SIV/GE and pGX10-SIV/dpol, and plasmids pGX10-SIV/VN and pGX10-SIV/TV (Group 3), the number of infectious cells after 12-24 weeks was less than 16 per million PBMCs; which means that the DNA vaccine used for this group was more efficient in inhibiting viral proliferation compared to plasmids pTV-SIV/GE and pTV-SIV/pol.

For the monkeys immunized with immunogenic plasmids pGX10-SIV/GE, pGX10-SIV/dpol, pGX10-SIV/VN and pGX10-SIV/TV together with adjuvant plasmid pGX10-hIL-12m (Group 4), number of infectious cells after 2 weeks are 10 times lower (4-512) than those of other groups (Group 1, Group 2+5 & Group 3), on average. Therefore, replication inhibition induced by immunization of immunogenic plasmids pGX10-SIV/GE, pGX10-SIV/dpol, pGX10-SIV/VN and pGX10-SIV/TV plus plasmid pGX10-hIL-12m seems the most effective. However, 12-24 weeks after infection, for one of the three monkeys, the number of infectious cells increased gradually. For this monkey, the number of infectious cells after 2 weeks was very low, and viral loads decline rate was lower than the other monkeys. Therefore, it was noted that the number of infectious cells after 20 weeks increased gradually. This corresponds with the report that decline of viral titer was important in determining the set-point titer (Staprans SI, *J. Virol.* 73,4829). However, for the other two monkeys, the number of infectious cells after 24 weeks was lower than 8 per million PBMCs, which shows

superior immunization efficacy compared with Group 2+5. Comparing results of Groups 3 and 4 with that of Group 2+5, vaccine immunized together with adjuvant plasmids obviously decreases number of SIVmac239-infectious cells in blood, which implies that immunization of DNA vaccine having adjuvant plasmid according to the present invention has excellent inhibition efficiency against replication of AIDS virus.

Experimental Example 4

Immune efficacy evaluation of plasmid according to the present invention-II

A. Immunization of monkey using immunogenic plasmid (Cf.: Fig. 26-Test Protocol)

Monkeys are immunized with immunogenic plasmids as in A of Experimental Example 3.

B. Immunization of monkey using immunogenic plasmid and adjuvant plasmid (Cf.: Fig. 26-Test Protocol)

Monkeys are immunized with immunogenic plasmids as in B of Experimental Example 3.

C. Determination of SIVmac239 RNA copies in plasma

Number of RNA copies in plasma was determined by Quantitative Competitive RT-PCR (Reverse Transcription PCR) method of BPRC (Biomedical Primate Research Centre, the Netherlands). In this method, RT-PCR was carried out using RNA

mixture of known reference SIVmac239 RNA and RNA separated from sample plasma as a template. If the number of RNA copies in plasma was larger than that of reference RNA, the plasma RNA was amplified; and otherwise, the reference RNA was amplified PCR. Size of the amplification product was measured by electrophoresis in agarose gel. The used reference RNA was the one which had been modified so that PCR product has a size larger or smaller than that of SIVmac239 RNA even when using the same primer. Accordingly, the discrepancy resulting from the size of the product should be compensated. More detailed description of this method was provided in a paper by Watson, et al. (*J. Virol.* 71,284-290, (1997)).

The number of RNA copies in plasma was another indication of viral infection degree. If SIV replicates actively, RNA was secreted to the plasma. Therefore, if the number of RNA copies in plasma was low, it means that RNA replication was inhibited properly (Fig. 28).

In Fig. 28, monkeys of Groups 1, 3 and 4 (control groups) showed more than 500,000 copies of SIV RNA in 1 ㎖ of plasma 4-24 weeks after infection. For monkeys immunized with plasmids pTV-SIV/GE and pTV-SIV/pol, which are used as controls, three of the five monkeys (Group 2+5) showed a reduction to 100,000 after 12 weeks and further reduction afterwards (16 weeks and 24 weeks). However, although the other two monkeys showed smaller number of viral copies after 12 weeks compared to infected monkeys (Group 1), they showed more than 100,000 copies after 16 weeks, which was not much different from that of infected monkeys (Groups 1, 3 and 4).

For Group 6, which was immunized with the regulatory gene according to the present invention, all the three monkeys showed a titer 10,000-100,000 after 12 and 16 weeks, which was more than 100 times less than that of infected monkeys (Group 1).

Consequently, immunization with immunogenic plasmids pGX10-SIV/GE and pGX10-SIV/pol together with the regulatory gene showed superior ability to inhibit viral replication compared with immunization with immunogenic plasmids pTV-SIV/GE and pTV-SIV/pol only.

Monkeys immunized with both regulatory gene and adjuvant plasmid pGX10-IL-12m DNA (Group 4) showed the lowest number of viral RAN copies after 2 weeks in all groups. Afterwards, the number of viral RNA copies was similar to that of Group 6. One monkey injected also with pGX10-hIL-12m showed very low viral titer after 2 weeks and increased titer after 16 weeks.

Experimental Example 5

Immune efficacy evaluation of plasmid according to the present invention-III

A. Immunization of monkey using immunogenic plasmid (Cf.: Fig. 26-Test Protocol)

Monkeys are immunized with immunogenic plasmids as in A of Experimental Example 3.

B. Immunization of monkey using immunogenic plasmid and adjuvant plasmid (Cf.: Fig. 26-Test Protocol)

Monkeys are immunized with immunogenic plasmids as in B of Experimental Example 3.

C. Determination of absolute CD4+ cell number in blood

Absolute number of CD4+ cells in unit volume of blood was determined, which was the most general method for evaluating AIDS progress in monkey and human. If monkey was infected with SIVmac239, number of CD4+ cells, which are the mainly infected cells, decreases gradually due to apoptosis. Reduction of CD4+ cell number causes dysfunction of monkey's immune system. This causes AIDS-associated disease, and consequently, the monkey dies. That was, reduction of absolute number of CD4+ cells means that immune functions of the host deteriorated, and that replication of SIVmac239 was not controlled effectively.

1 week, 2 weeks, 4 weeks, 6 weeks, 8 weeks, 12 weeks, 16 weeks, 20 weeks, 24 weeks and 28 weeks after SIVmac239 infection, the number of CD4+ cells in PBLs (peripheral blood lymphocytes) of monkey blood was determined by FACS (fluorescent automated cell sorter; Becton-Dickinson, FACScan) (Bjorn, et al., *J. Virol.* 72,7846, (1998)). The results are shown in Fig. 29.

In Fig. 29, absolute number of CD4+ T cells in 1 $\mu\ell$ of blood was presented as a percentage of the number before infection (100%). The initial number of CD4+ T cells was determined before SIV infection at more than two time points. Fig. 29 shows that one monkey in Group 1 showed a gradual decrease in the number of CD4+ cells, and reach 50% of initial value % after 28 weeks. One of the five monkeys immunized with immunogenic plasmids pTV-SIV/GE and pTV-SIV/pol showed a drop than 50% at this point (after 28 weeks). However, all monkeys in Group 3 and Group 4, wherein the regulatory gene was used according to the present invention, showed a normal number of CD4+ cells. This means that immunization of immunogenic plasmids pGX10-SIV/GE and pGX10-SIV/dpol together with regulatory gene was the

most efficient in preventing decrease of CD4+ cell numbers due to SIVmac239 infection.

Experimental Example 6

Immune efficacy of evaluation of plasmid according to the present invention-IV

A. Immunization of monkey using immunogenic plasmid (Cf.: Fig. 26-Test Protocol)

Monkeys are immunized with immunogenic plasmids as in A of Experimental Example 3.

B. Immunization of monkey using immunogenic plasmid and adjuvant plasmid (Cf.: Fig. 26-Test Protocol)

Monkeys are immunized with immunogenic plasmids as in B of Experimental Example 3.

C. Measurement of gag-specific T cell reaction induced by SIVmac239 DNA immunization

ELISPOT test was used to measure T cell immune reaction induced by SIV DNA vaccine, wherein PBMCs are stimulated by a Gag peptide pool and the number of PBMCs that secrete INF-γ was determined. In a 96-well plate (U-cytech), 5 μg (100 μℓ in PBS) of anti-IFN-γ mAb per well was let stand for 15hr at 4℃. It was then washed 6 times with PBST (PBS + 0.05% Tween-20). After adding 200 μℓ of 2%

BSA/PBS per well for 1hr at 37℃ to block sites not bound to mAb, 100 ㎕ of ELISPOT test medium (45% RPMI1640, 45% XVIVO, 10% fetal bovin serum, 1% antibiotics) including 2×10^5 PBMCs (purification method of PBMC was described in B of Experimental Example 3) was added per well. Then, SIV gag p15 and p26 overlapping peptide (NIBSC, cat. number ARP714.1-22 and EVA776.1-776.14) was added in the 10 ㎕ ELISPOT test medium, so that the final peptide concentration becomes 1 ㎍/㎖. The plate was incubated for 24hr in culture medium under CO_2 atmosphere. After removing the medium, cells attached to the plate are lysed with 200 ㎕ of ice-cold deionized water. After washing the plate 10 times with PBST, 1 ㎍ (100 ㎕ in PBS+1% BSA) of rabbit polyclonal biotinylated anti-IFN-γ detector Ab (U-cytech) was added to each well. After incubating for 1hr at 37℃, the plate was washed 7 times with PBST, then 50 ㎕ of gold-labeled anti-biotin IgG (U-cytech) was added to each well. After a further incubation for 1hr at 37℃, the plate was washed 10 times with PBST and 35 ㎕ of activator mixture (U-cytech) was added to each well. The plate was let stand for 30min. After washing the plate with water, the number of spots formed was counted within 48 hour.

The results are shown in Fig. 30. In Fig. 30, the number of T cells per million PBMCs that secrete IFN-γ due to stimulation by gag peptide was shown. This represents the number of SIVmac239 gag-specific T cells. A larger number means that a stronger gag-specific T cell immune responses were induced.

As shown in Fig. 30, a gag-specific immune response was induced by the group immunized with immunogenic plasmids pTV-SIV/GE and pTV-SIV/pol (Group 5) and the group immunized with immunogenic plasmids pGX10-SIV/GE, pGX10-SIV/pol, pGX10-SIV/VN and pGX10-SIV/TV according to the present

invention (Group 3), and there was no difference in strength of the response. This means that pTV2 and pGX10 vectors induce similar T cell immune response in monkey. Accordingly, the superior viral replication inhibition effect generated by immunization with immunogenic plasmids pGX10-SIV/GE, pGX10-SIV/pol, pGX10-SIV/VN and pGX10-SIV/TV according to the present invention seems to be caused by the presence of regulatory gene, rather than by the difference in vector pGX10.

Also, because no difference was detected in gag-specific T cell responses of Group 5 and Group 3, the hypothesis of Cafaro A, saying that quantitative difference in immunostimulatory sequence such as CpG motif caused by difference of DNA doses increases immune reaction, and hence results in difference in protection efficiency (Cafaro A., et al., *Vaccine* 19, 2867, (2001)) can be excluded. When adjuvant plasmid pGX10-hIL-12m DNA was co-immunized , the higher gag-specific T cell responses were generated compared with immunization without it (Group 3).

Experimental Example 7

Confirmation of expression of immunogenic plasmids pGX10-SIV/pol and pGX10-SIV/GE according to the present invention (Fig. 31)

3.0×10^5 HeLa cells were placed on a 60mM dish (Felcon). After adding 4.5 ㎖ of DMEM (10% FCS) culture medium, it was incubated for 12hr at 37℃ under 5% CO_2. 10 ㎍ of DNA (pGX10, pTV2, pGX10-SIV/GE, pGX10-SIV/pol and pTV2-SIV/GE) was mixed with 250 ㎕ of 0.2M $CaCl_2$ solution and stirred well. This solution was added to 250 ㎕ 2X HBS solution dropwise. After letting stand for

15min at room temperature, the solution was stirred slightly with a pipette and spread on the cultured HeLa cells. After culturing for 60 more hours at 37℃ under 5% CO_2, the culture medium was removed. After washing with PBS, the cells are harvested for SDS-PAGE (SDS-polyacrylamide gel electrophorsis). After performing the SDS-page the proteins on the gel was transferred to nitrocellulose membrane (Schleicher & Schuell) after PAGE (refer to Molecular Cloning, 18. 64-66 for transfer condition and buffer composition). After blocking the membrane for 2hr at room temperature with blocking solution (5% skim milk/TBS), it was washed with washing buffer (TBS + 0.05% tween-20) three times in a shaker for 5min each. Blocking buffer containing 1/3000 (v/v) of SIVmac antiserum (NIBSC APR416) was reacted for 3hr at room temperature, and washed three times with washing buffer for 10min. Blocking buffer containing 1/3000 (v/v) of HRP-conjugated anti-human IgG (Sigma) was reacted with the membrane for 2hr at room temperature, and washed 4 times with washing buffer. After washing the membrane with distilled water 5 times, 10 ㎖ of 1:1 Luminol/Enhancer solution (Pierce) and Stable Peroxide Solution (Pierce) was added to initiate a peroxidase reaction. After exposing the product for 10min on X-ray film (AGFA), the film was developed to obtain the expression profile.

As shown in Fig.31, pGX10-SIV/GE expressed SIV env and gag proteins well, and pGX10-SIV/pol expressed RT-INT polyprotein well. For pTV2-SIV/GE, gag and env protein expression was significantly lower than pGX10-SIV/GE. This means that pGX10 vector was superior in in-vitro expression efficiency, compared with pTV2.

Experimental Example 8

Identification of expression of immunogenic plasmids pGX10-SIV/VN and pGX10-SIV/VNTV according to the present invention (Fig. 33)

Plasmids pGX10, pGX10-SIV/VN and pGX10-SIV/VNTV are transfected into HeLa cells. SIV nef polyclonal Ab (NIBSC APR444) was used to detect vif-nef protein, and HRP-conjugated anti-rabbit IgG was used as secondary antibody. Other details are the same as those of Experimental Example 7.

As shown in Fig. 33, pGX10-SIV/VN and pGX10-SIV/VNTV expressed vif-nef protein well in HeLa cell.

Experimental Example 9

Identification of expression of immunogenic plasmid pGX10-SIV/TV according to the present invention (Fig. 34)

Plasmids pGX10 and pGX10-SIV/TV are transfected into HeLa cells. SIV tat polyclonal Ab (NIBSC APR4006) was used to detect tat-vpx protein, and HRP-conjugated anti-rabbit IgG was used as secondary antibody. Other details are the same as those of Experimental Example 7.

As shown in Fig. 34, pGX10-SIV/TV expressed tat-vpx protein well in HeLa cell.

Toxicity Test 1

Administration of HIV DNA vaccine to chimpanzee

HIV DNA vaccine containing immunogenic plasmids pGX10-HIV/pol, pGX10-HIV/GE and pGX10-HIV/VNTV and adjuvant plasmid pGX10-hIL-12m (2 ㎎

each) according to the present invention was administered to chimpanzees chronically infected with HIV-1 (HIV IIIB), through gluteus maximus (or deltoid muscle) injection. One chimpanzee was injected 4 times at one month intervals, and the other chimpanzee was injected 4 times at one month interval and after 4 months period, injected again 5 times at one month interval.

Physical examination including clinical signs, weight and body temperature, haematology and urinalysis are carried out for one year after initial immunization. No anomaly was observed.

Toxicity Test 2

Administration of HIV DNA vaccine to human

HIV DNA vaccine containing immunogenic plasmids pGX10-HIV/pol, pGX10-HIV/GE and pGX10-HIV/VNTV and adjuvant plasmid pGX10-hIL-12m (1 ㎎ each) according to the present invention was administered 8 Ukrainian patients infected with HIV-1. Four persons are injected 6 times at 2 months intervals; two persons were injected 5 times at 2 month intervals; and the remaining two persons were injected 4 times at 2 month intervals, through intramuscular injection. Injection were alternately administered to gluteus maximus and deltoid muscles.

Physical examination including clinical signs, weight and body temperature, haematology and urinalysis are carried out for one year after initial immunization. No anomaly was observed.

INDICATIONS RELATING TO DEPOSITED MICROORGANISM OR OTHER BIOLOGICAL MATERIAL

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What Is Claimed Is:

1. A vector pGX10.

2. A plasmid pGX10-SIV/GE.

3. A plasmid comprising the vector pGX10 and the SIVmac239 pol gene encoding reverse transcriptase and integrase and a DNA sequence encoding a signal peptide of secretory protein fused to the 3' end of the SIVmac239 pol gene which are operably linked to the vector.

4. The plasmid of claim 3, in which said pol gene is mutated so that the enzymatic activity of integrase can be inhibited.

5. The plasmid of claim 4, in which the mutation inhibiting the enzymatic activity of integrase comprises the deletion of nucleotides 5130-5132 site and the substitution of nucleotides 5133-5135 site for a serine codon.

6. The plasmid of claim 3, in which the DNA sequence encoding a signal peptide of secretory protein comprises the DNA sequence encoding a signal peptide of glycoprotein.

7. The plasmid of claim 6, in which the DNA sequence encoding a signal peptide of glycoprotein comprises one derived from herpes simplex virus.

8. The plasmid of claim 3 which is pGX10-SIV/dpol.

9. A plasmid comprising the SIVmac239 vif gene and a DNA sequence encoding a signal peptide of secretory protein and the SIVmac239 nef gene fused to the 3' and 5' ends of the SIVmac239 vif gene, respectively.

10. The plasmid of claim 9, in which the DNA sequence encoding a signal peptide of secretory protein fused to the 3' end of the SIVmac239 vif gene comprises the DNA sequence encoding a signal peptide of glycoprotein.

11. The plasmid of claim 10, in which the DNA sequence encoding a signal peptide of glycoprotein comprises one derived from herpes simplex virus.

12. The plasmid of claim 9, in which the mutation of the SIVmac239 nef gene fused to the 5' end of the SIVmac239 vif gene comprises the deletion of codons for Arg137 and Arg138.

13. The plasmid of claim 12, in which the mutation of the SIVmac239 nef gene fused to the 5' end of the SIVmac239 vif gene comprises the deletion of codons for Arg137 and Arg138.

14. The plasmid of claim 9 which is pGX10-SIV/VN.

15. A plasmid comprising any one of genes having from exon 1 to the full length of the SIVmac239 tat gene, and the DNA sequence encoding a signal peptide of secretory protein and the SIVmac239 vpx gene fused to the 3' and 5' ends of the SIVmac239 tat gene, respectively.

16. The plasmid of claim 15, in which the signal sequence DNA of secretory protein fused to the 3' end of any one of genes having from exon 1 to a full length of the SIVmac239 tat gene comprises the DNA sequence encoding a signal peptide of glycoprotein.

17. The plasmid of claim 16, in which the DNA sequence encoding a signal peptide of glycoprotein comprises one derived from herpes simplex virus.

18. The plasmid of claim 15, in which the SIVmac239 tat gene is exon 1 of the SIVmac239 tat gene.

19. The plasmid of claim 15, which is pGX10-SIV/TV.

20. A plasmid comprising (i) the SIVmac239 vif gene and the DNA sequence encoding a signal peptide of secretory protein and the SIVmac239 nef gene fused to the 3' and 5' ends of the SIVmac239 vif gene, respectively, and (ii) any one of genes having from exon 1 to a full length of the SIVmac239 tat gene and the DNA sequence encoding a signal peptide of secretory protein and the SIVmac239 vpx gene fused to the 3' and 5' ends of the SIVmac239 tat gene, respectively.

21. The plasmid of claim 20, in which the signal sequence DNA of secretory protein fused to the 3' end of the SIVmac239 vif gene comprises the DNA sequence encoding a signal peptide of glycoprotein.

22. The plasmid of claim 21, in which the DNA sequence encoding a signal peptide of glycoprotein comprises one derived from herpes simplex virus.

23. The plasmid of claim 20, in which the mutation of the SIVmac239 nef gene fused to the 5' end of the SIVmac239 vif gene comprises the deletion of codons for Arg137 and Arg138.

24. The plasmid of claim 23, in which the mutation of the SIVmac239 nef gene fused to the 5' end of the SIVmac239 vif gene comprises the deletion of codons for Arg137 and Arg138.

25. The plasmid of claim 20, in which the signal sequence DNA of secretory protein fused to the 3' end of any one of genes having from exon 1 to a full length of the SIVmac239 tat gene comprises the DNA sequence encoding a signal peptide of glycoprotein.

26. The plasmid of claim 25, in which the DNA sequence encoding a signal peptide of glycoprotein comprises one derived from herpes simplex virus.

27. The plasmid of claim 25, in which the SIVmac239 tat gene is exon 1 of the SIVmac239 tat gene.

28. The plasmid of claim 25, which is pGX10-SIV/VNTV.

29. The plasmid of claim 25, which is pGX10-SIV/TVVN.

30. A plasmid pGX10-HIV/GE.

31. A plasmid comprising the vector pGX10, and the HIV-1 pol gene encoding reverse transcriptase and integrase and the DNA sequence encoding a signal peptide of secretory protein fused to the 3' end of the HIV-1 pol gene which are operably linked to the vector.

32. The plasmid of claim 31, in which said pol gene is mutated so that the enzymatic activity of integrase can be inhibited.

33. The plasmid of claim 32, in which the mutation inhibiting the enzymatic activity of integrase comprises the deletion of nucleotides 5130-5132 and the substitution of nucleotides 5133-5135 for a serine codon.

34. The plasmid of claim 31, in which the signal sequence DNA of secretory protein comprises the DNA sequence encoding a signal peptide of glycoprotein.

35. The plasmid of claim 34, in which the DNA sequence encoding a signal peptide of glycoprotein comprises one derived from herpes simplex virus.

36. The plasmid of claim 31 which is pGX10-HIV/dpol.

37. A plasmid comprising the HIV-1 vif gene and the DNA sequence encoding a signal peptide of secretory protein and the HIV-1 nef gene fused to the 3' and 5' ends of the HIV-1 vif gene, respectively.

38. The plasmid of claim 37, in which the signal sequence DNA of secretory protein fused to the 3' end of the HIV-1 vif gene comprises the DNA sequence encoding a signal peptide of glycoprotein.

39. The plasmid of claim 38, in which the DNA sequence encoding a signal peptide of glycoprotein comprises one derived from herpes simplex virus.

40. The plasmid of claim 37, in which the mutation of the HIV-1 nef gene fused to the 5' end of the HIV-1 vif gene comprises the deletion of codons for Arg137 and Arg138.

41. The plasmid of claim 40, in which the mutation of the HIV-1 nef gene fused to the 5' end of the HIV-1 vif gene comprises the deletion of codons for Arg137 and Arg138.

42. The plasmid of claim 37, which is pGX10-HIV/VN.

43. A plasmid comprising any one of genes having from exon 1 to a full length of HIV-1 tat gene and the DNA sequence encoding a signal peptide of secretory protein and HIV-1 vpx gene fused to the 3' and 5' ends of the HIV-1 tat gene, respectively.

44. The plasmid of claim 43, in which the signal sequence DNA of secretory protein fused to the 3' end of any one of genes having from exon 1 to a full length of HIV-1 tat gene comprises the DNA sequence encoding a signal peptide of glycoprotein.

45. The plasmid of claim 44, in which the DNA sequence encoding a signal peptide of glycoprotein comprises one derived from herpes simplex virus.

46. The plasmid of claim 43, in which the HIV-1 tat gene is exon 1 of the HIV-1 tat gene.

47. The plasmid of claim 43, which is pGX10-HIV/TV.

48. A plasmid comprising (i) HIV-1 vif gene and the DNA sequence encoding a signal peptide of secretory protein and HIV-1 nef gene fused to the 3' and 5' ends of the HIV-1 vif gene, respectively, and (ii) any one of genes having from exon

1 to a full length of HIV-1 tat gene and the DNA sequence encoding a signal peptide of secretory protein and HIV-1 vpx gene fused to the 3' and 5' ends of the HIV-1 tat gene, respectively.

49. The plasmid of claim 48, in which the signal sequence DNA of secretory protein fused to the 3' end of the HIV-1 vif gene comprises the DNA sequence encoding a signal peptide of glycoprotein.

50. The plasmid of claim 49, in which the DNA sequence encoding a signal peptide of glycoprotein comprises one derived from herpes simplex virus.

51. The plasmid of claim 48, in which the mutation of the HIV-1 nef gene fused to the 5' end of the HIV-1 vif gene comprises the deletion of codons for Arg137 and Arg138.

52. The plasmid of claim 51, in which the mutation of the HIV-1 nef gene fused to the 5' end of the HIV-1 vif gene comprises the deletion of codons for Arg137 and Arg138.

53. The plasmid of claim 48, in which the signal sequence DNA of secretory protein fused to the 3' end of any one of genes having from exon 1 to a full length of HIV-1 tat gene comprises the DNA sequence encoding a signal peptide of glycoprotein.

54. The plasmid of claim 53, in which the DNA sequence encoding a signal peptide of glycoprotein comprises one derived from herpes simplex virus.

55. The plasmid of claim 48, in which the HIV-1 tat gene is exon 1 of the tat gene.

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56. The plasmid of claim 48, which is pGX10-HIV/VNTV.

57. The plasmid of claim 48, which is pGX10-HIV/TVVN.

10 58. A plasmid pGX10-hIL-12m.

59. A DNA vaccine composition for prophylaxis or treatment of AIDS comprising (i) plasmid pGX10-SIV/GE and (ii) plasmid comprising the vector pGX10 and the SIVmac239 pol gene encoding reverse transcriptase and integrase and the DNA 15 sequence encoding a signal peptide of secretory protein fused to the 3' end of the SIVmac239 pol gene which are operably linked to the vector.

60. The DNA vaccine composition of claim 59, which further comprises plasmid pGX10-hIL-12m.

20

61. A DNA vaccine composition for prophylaxis or treatment of AIDS comprising (i) plasmid comprising the SIVmac239 gag, dpol, env and rev genes, (ii) plasmid comprising the SIVmac239 pol gene encoding reverse transcriptase and invertase and the DNA sequence encoding a signal peptide of secretory protein fused to

the 3' end of the SIVmac239 pol gene, and (iii) plasmid comprising the SIVmac239 vif gene and the DNA sequence encoding a signal peptide of secretory protein and the SIVmac239 nef gene fused to the 3' and 5' ends of the vif gene, respectively, or plasmid comprising any one of genes having from exon 1 to a full length of the SIVmac239 tat gene and the DNA sequence encoding a signal peptide of secretory protein and the SIVmac239 vpx gene fused to the 3' and 5' ends of the SIVmac239 tat gene, respectively.

62. The DNA vaccine composition of claim 61, in which the plasmid comprising the SIVmac239 gag, dpol, env and rev genes is pTV-SIV/GE or pGX10-SIV/GE.

63. The DNA vaccine composition of claim 61, in which the pol gene in the plasmid comprising the SIVmac239 pol gene encoding reverse transcriptase and invertase and the DNA sequence encoding a signal peptide of secretory protein fused to the 3' end of the pol gene is mutated so that the enzymatic activity of integrase can be inhibited.

64. The DNA vaccine composition of claim 63, in which the mutation inhibiting the enzymatic activity of integrase comprises the deletion of nucleotides 5130-5132 and the substitution of nucleotides 5133-5135 for a serine codon.

65. The DNA vaccine composition of claim 61, in which the plasmid comprising the SIVmac239 pol gene encoding reverse transcriptase and invertase and

the DNA sequence encoding a signal peptide of secretory protein fused to the 3' end of the SIVmac239 pol gene is pTV-SIV/dpol or pGX10-SIV/dpol.

66. The DNA vaccine composition of claim 61, which further comprises plasmid pGX10-hIL-12m.

67. A DNA vaccine composition for prophylaxis or treatment of AIDS comprising (i) plasmid comprising the SIVmac239 gag, dpol, env and rev genes, (ii) plasmid comprising the SIVmac239 pol gene encoding reverse transcriptase and invertase and the DNA sequence encoding a signal peptide of secretory protein fused to the 3' end of the SIVmac239 pol gene, (iii) plasmid comprising the SIVmac239 vif gene and the DNA sequence encoding a signal peptide of secretory protein and the SIVmac239 nef gene fused to the 3' and 5' ends of the vif gene, respectively, and (iv) plasmid comprising any one of genes having from exon 1 to a full length of the SIVmac239 tat gene and the DNA sequence encoding a signal peptide of secretory protein and the SIVmac239 vpx gene fused to the 3' and 5' ends of the SIVmac239 tat gene, respectively.

68. The DNA vaccine composition of claim 67, in which the plasmid comprising the SIVmac239 gag, dpol, env and rev genes is pTV-SIV/GE or pGX10-SIV/GE.

69. The DNA vaccine composition of claim 67, in which the pol gene in the plasmid comprising the SIVmac239 pol gene encoding reverse transcriptase and

invertase and the DNA sequence encoding a signal peptide of secretory protein fused to the 3' end of the pol gene is mutated so that the enzymatic activity of integrase can be inhibited.

70. The DNA vaccine composition of claim 69, in which the mutation inhibiting the enzymatic activity of integrase comprises the deletion of nucleotides 5130-5132 site and the substitution of nucleotides 5133-5135 site for a serine codon.

71. The DNA vaccine composition of claim 67, in which the plasmid comprising the SIVmac239 pol gene encoding reverse transcriptase and invertase and the DNA sequence encoding a signal peptide of secretory protein fused to the 3' end of the SIVmac239 pol gene is pTV-SIV/dpol or pGX10-SIV/dpol.

72. The DNA vaccine composition of claim 67, which further comprises plasmid pGX10-hIL-12m.

73. A DNA vaccine composition for prophylaxis or treatment of AIDS comprising (i) plasmid comprising the SIVmac239 gag, dpol, env and rev genes, (ii) plasmid comprising the SIVmac239 pol gene encoding reverse transcriptase and invertase and the DNA sequence encoding a signal peptide of secretory protein fused to the 3' end of the SIVmac239 pol gene, and (iii) plasmid comprising (a) SIVmac239 vif gene and the DNA sequence encoding a signal peptide of secretory protein and the SIVmac239 nef gene fused to the 3' and 5' ends of the SIVmac239 vif gene, respectively, and (b) any one of genes having from exon 1 to a full length of the

SIVmac239 tat gene and the DNA sequence encoding a signal peptide of secretory protein and the SIVmac239 vpx gene fused to the 5' end of the SIVmac239 tat gene, respectively.

74. The DNA vaccine composition of claim 73, in which the plasmid comprising the SIVmac239 gag, dpol, env and rev genes is pTV-SIV/GE or pGX10-SIV/GE.

75. The DNA vaccine composition of claim 73, in which the pol gene in plasmid comprising the SIVmac239 pol gene encoding reverse transcriptase and invertase and the DNA sequence encoding a signal peptide of secretory protein fused to the 3' end of the SIVmac239 pol gene is mutated so that the enzymatic activity of integrase can be inhibited.

76. The DNA vaccine composition of claim 75, in which the mutation inhibiting the enzymatic activity of integrase comprises the deletion of nucleotides 5130-5132 site and the substitution of nucleotides 5133-5135 site for a serine codon.

77. The DNA vaccine composition of claim 73, in which the plasmid comprising the SIVmac239 pol gene encoding reverse transcriptase and invertase and the DNA sequence encoding a signal peptide of secretory protein fused to the 3' end of the SIVmac239 pol gene is pTV-SIV/dpol or pGX10-SIV/dpol.

78. The DNA vaccine composition of claim 73, which further comprises

plasmid pGX10-hIL-12m.

79. A DNA vaccine composition for prophylaxis or treatment of AIDS comprising (i) plasmid pGX10-HIV/GE and (ii) plasmid comprising the vector pGX10 and the HIV-1 pol gene encoding reverse transcriptase and integrase and the DNA sequence encoding a signal peptide of secretory protein fused to the 3' end of the HIV-1 pol gene which are operably linked to the vector.

80. The DNA vaccine composition of claim 79, which further comprises plasmid pGX10-hIL-12m.

81. A DNA vaccine composition for prophylaxis or treatment of AIDS comprising (i) plasmid comprising HIV-1 gag, dpol, env and rev genes, (ii) plasmid comprising the HIV-1 pol gene encoding reverse transcriptase and invertase and the DNA sequence encoding a signal peptide of secretory protein fused to the 3' end of the HIV-1 pol gene, (iii) plasmid comprising HIV-1 vif gene and the DNA sequence encoding a signal peptide of secretory protein and HIV-1 nef gene fused to the 3' and 5' ends of the vif gene, respectively, or plasmid comprising any one of genes having from exon 1 to a full length of the HIV-1 tat gene and the DNA sequence encoding a signal peptide of secretory protein and HIV-1 vpx gene fused to the 3' and 5' ends of the HIV-1 tat gene, respectively.

82. The DNA vaccine composition of claim 81, in which the plasmid comprising the HIV-1 gag, dpol, env and rev genes is pTV-HIV/GE or pGX10-

HIV/GE.

83. The DNA vaccine composition of claim 81, in which the pol gene in plasmid comprising the HIV-1 pol gene encoding reverse transcriptase and invertase and the DNA sequence encoding a signal peptide of secretory protein fused to the 3' end of the pol gene is mutated so that the enzymatic activity of integrase can be inhibited.

84. The DNA vaccine composition of claim 83, in which the mutation inhibiting the enzymatic activity of integrase comprises the deletion of nucleotides 5130-5132 site and the substitution of nucleotides 5133-5135 site for a serine codon.

85. The DNA vaccine composition of claim 81, in which the plasmid comprising the HIV-1 pol gene encoding reverse transcriptase and invertase and the DNA sequence encoding a signal peptide of secretory protein fused to the 3' end of the HIV-1 pol gene is pTV-HIV/dpol or pGX10-HIV/dpol.

86. The DNA vaccine composition of claim 81, which further comprises plasmid pGX10-hIL-12m.

87. A DNA vaccine composition for prophylaxis or treatment of AIDS comprising (i) plasmid comprising the HIV-1 gag, dpol, env and rev genes, (ii) plasmid comprising HIV-1 pol gene encoding reverse transcriptase and invertase and the DNA sequence encoding a signal peptide of secretory protein fused to the 3' end of the HIV-

1 pol gene, and (iii) plasmid comprising (a) HIV-1 vif gene and the DNA sequence encoding a signal peptide of secretory protein and HIV-1 nef gene fused to the 3' and 5' ends of the vif gene, respectively, and (b) any one of genes having from exon 1 to a full length of HIV-1 tat gene and the DNA sequence encoding a signal peptide of secretory protein and HIV-1 vpx gene fused to the 5' end of the HIV-1 tat gene, respectively.

88. The DNA vaccine composition of claim 87, in which the plasmid comprising HIV-1 gag, dpol, env and rev genes is pTV-HIV/GE or pGX10-HIV/GE.

89. The DNA vaccine composition of claim 87, in which the pol gene in plasmid comprising HIV-1 pol gene encoding reverse transcriptase and invertase and the DNA sequence encoding a signal peptide of secretory protein fused to the 3' end of the HIV-1 pol gene is mutated so that the enzymatic activity of integrase can be inhibited.

90. The DNA vaccine composition of claim 89, in which the mutation inhibiting the enzymatic activity of integrase comprises the deletion of nucleotides 5130-5132 site and the substitution of nucleotides 5133-5135 site for a serine codon.

91. A DNA vaccine composition for prophylaxis or treatment of AIDS comprising (i) plasmid comprising HIV-1 gag, dpol, env and rev genes, (ii) plasmid comprising the HIV-1 pol gene encoding reverse transcriptase and invertase and the DNA sequence encoding a signal peptide of secretory protein fused to the 3' end of the

HIV-1 pol gene, (iii) plasmid comprising HIV-1 vif gene and the DNA sequence encoding a signal peptide of secretory protein and HIV-1 nef gene fused to the 3' and 5' ends of the vif gene, respectively, and (iv) plasmid comprising any one of genes having from exon 1 to a full length of the HIV-1 tat gene and the DNA sequence encoding a signal peptide of secretory protein and HIV-1 vpx gene fused to the 3' and 5' ends of the HIV-1 tat gene, respectively.

92. The DNA vaccine composition of claim 91, in which the plasmid comprising the HIV-1 gag, dpol, env and rev genes is pTV-HIV/GE or pGX10-HIV/GE.

93. The DNA vaccine composition of claim 91, in which the pol gene in plasmid comprising the HIV-1 pol gene encoding reverse transcriptase and invertase and the DNA sequence encoding a signal peptide of secretory protein fused to the 3' end of the pol gene is mutated so that the enzymatic activity of integrase can be inhibited.

94. The DNA vaccine composition of claim 93, in which the mutation inhibiting the enzymatic activity of integrase comprises the deletion of nucleotides 5130-5132 site and the substitution of nucleotides 5133-5135 site for a serine codon.

95. The DNA vaccine composition of claim 91, in which the plasmid comprising the HIV-1 pol gene encoding reverse transcriptase and invertase and the DNA sequence encoding a signal peptide of secretory protein fused to the 3' end of the

HIV-1 pol gene is pTV-HIV/dpol or pGX10-HIV/dpol.

96. The DNA vaccine composition of claim 91, which further comprises plasmid pGX10-hIL-12m.

97. A DNA vaccine composition for prophylaxis or treatment of AIDS comprising (i) plasmid comprising the HIV-1 gag, dpol, env and rev genes, (ii) plasmid comprising HIV-1 pol gene encoding reverse transcriptase and invertase and the DNA sequence encoding a signal peptide of secretory protein fused to the 3' end of the HIV-1 pol gene, and (iii) plasmid comprising (a) HIV-1 vif gene and the DNA sequence encoding a signal peptide of secretory protein and HIV-1 nef gene fused to the 3' and 5' ends of the vif gene, respectively, and (b) any one of genes having from exon 1 to a full length of HIV-1 tat gene and the DNA sequence encoding a signal peptide of secretory protein and HIV-1 vpx gene fused to the 5' end of the HIV-1 tat gene, respectively.

98. The DNA vaccine composition of claim 97, in which the plasmid comprising HIV-1 gag, dpol, env and rev genes is pTV-HIV/GE or pGX10-HIV/GE.

99. The DNA vaccine composition of claim 97, in which the pol gene in plasmid comprising HIV-1 pol gene encoding reverse transcriptase and invertase and the DNA sequence encoding a signal peptide of secretory protein fused to the 3' end of the HIV-1 pol gene is mutated so that the enzymatic activity of integrase can be inhibited.

100. The DNA vaccine composition of claim 99, in which the mutation inhibiting the enzymatic activity of integrase comprises the deletion of nucleotides 5130-5132 site and the substitution of nucleotides 5133-5135 site for a serine codon.

101. The DNA vaccine composition of claim 97, in which the plasmid comprising the HIV-1 pol gene encoding reverse transcriptase and invertase and the DNA sequence encoding a signal peptide of secretory protein fused to the 3' end of the HIV-1 pol gene is pTV-HIV/dpol or pGX10-HIV/dpol.

102. The DNA vaccine composition of claim 97, which further comprises plasmid pGX10-hIL-12m.

FIG. 1A

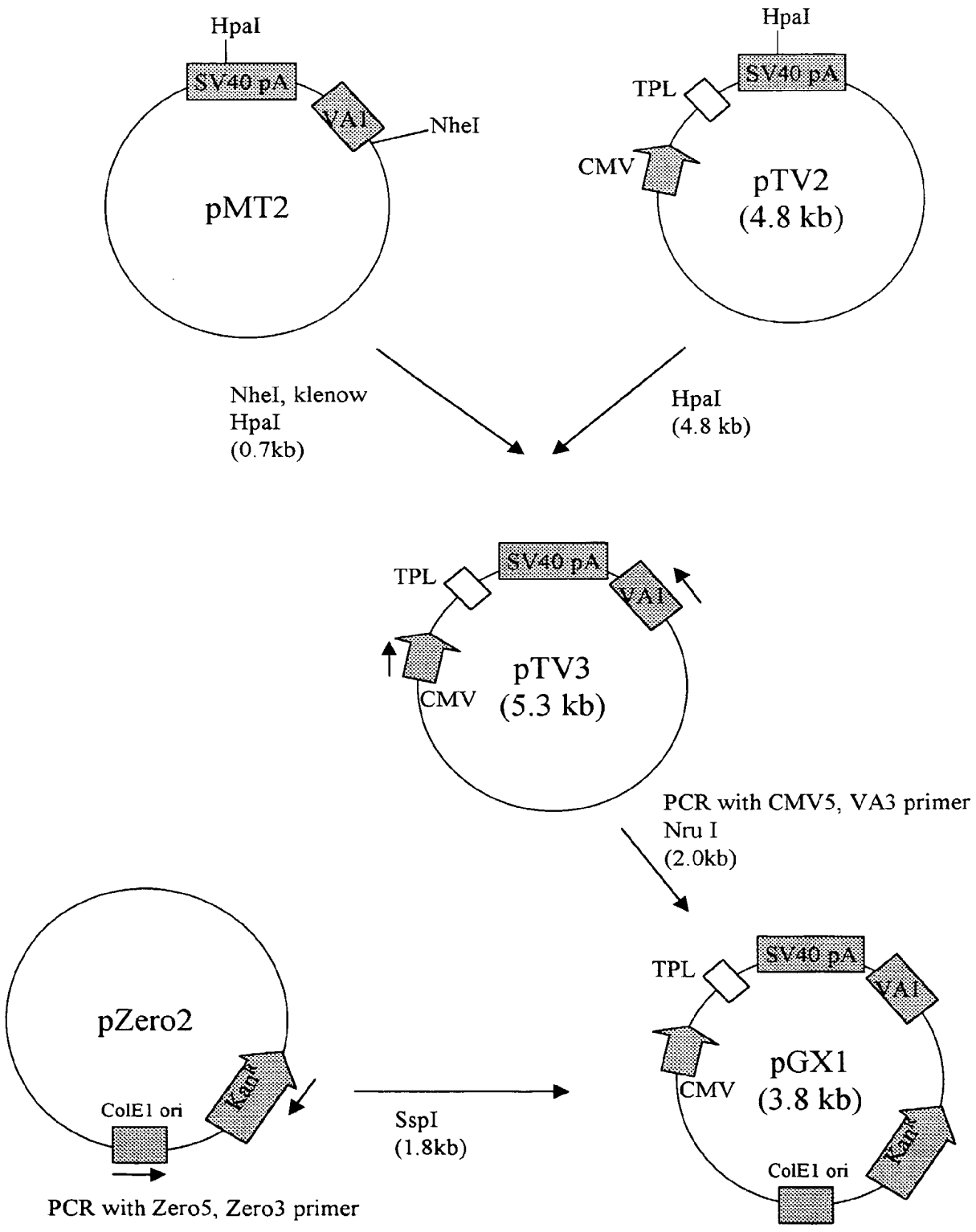

HpaI
SV40 pA
VA1
NheI
pMT2
HpaI
TPL
SV40 pA
CMV
pTV2
(4.8 kb)
NheI, klenow
HpaI
(0.7kb)
HpaI
(4.8 kb)
TPL
SV40 pA
VA1
CMV
pTV3
(5.3 kb)
PCR with CMV5, VA3 primer
Nru I
(2.0kb)
pZero2
ColE1 ori
Kan
SspI
(1.8kb)
TPL
SV40 pA
VA1
CMV
pGX1
(3.8 kb)
ColE1 ori
Kan
PCR with Zero5, Zero3 primer

FIG. 1B

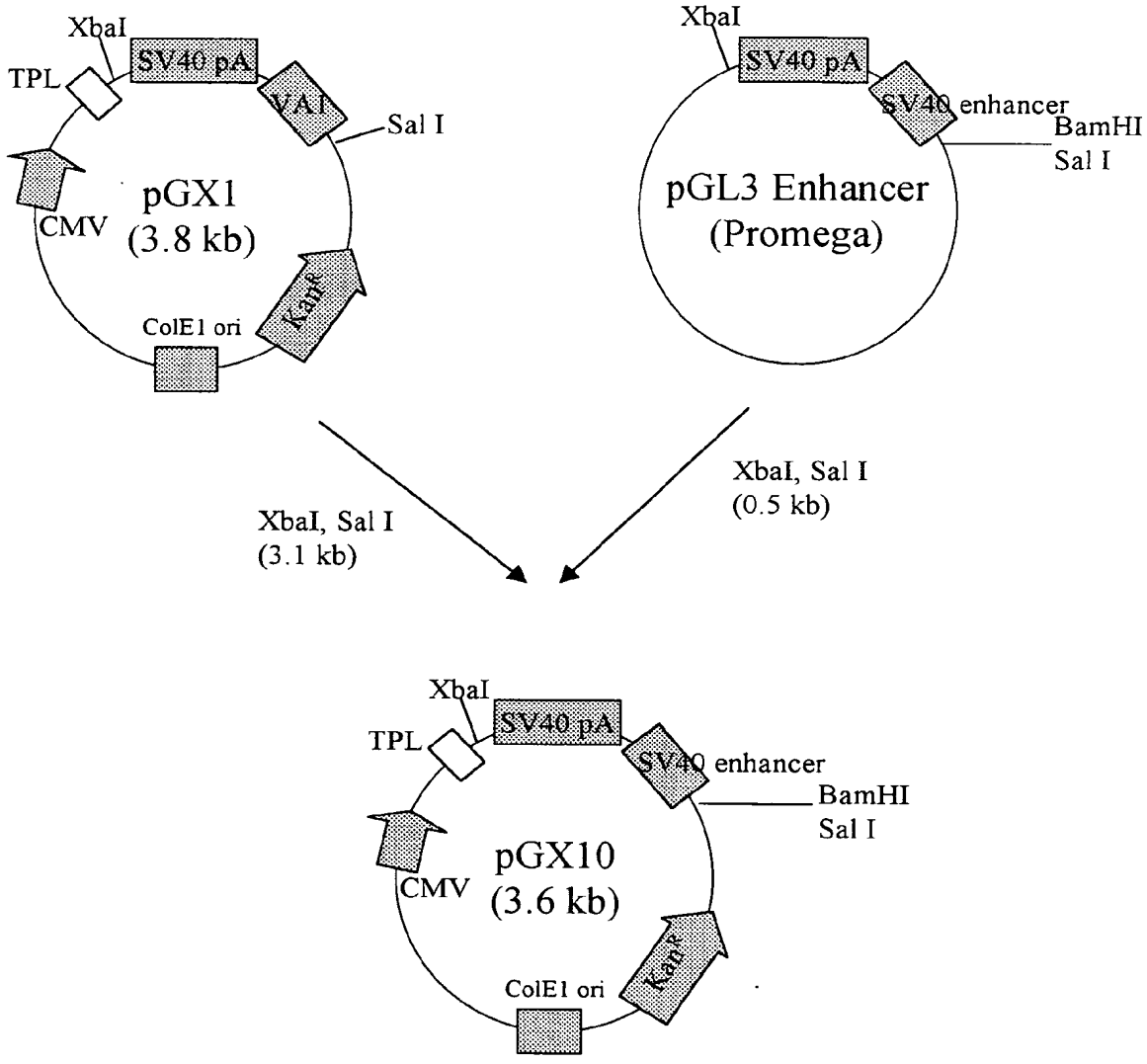

XbaI
SV40 pA
TPL
VA I
Sal I
CMV
pGX1
(3.8 kb)
ColE1 ori
Kan^R
XbaI
SV40 pA
SV40 enhancer
BamHI
Sal I
pGL3 Enhancer
(Promega)
XbaI, Sal I
(3.1 kb)
XbaI, Sal I
(0.5 kb)
XbaI
SV40 pA
TPL
SV40 enhancer
BamHI
Sal I
CMV
pGX10
(3.6 kb)
ColE1 ori
Kan^R

FIG. 2

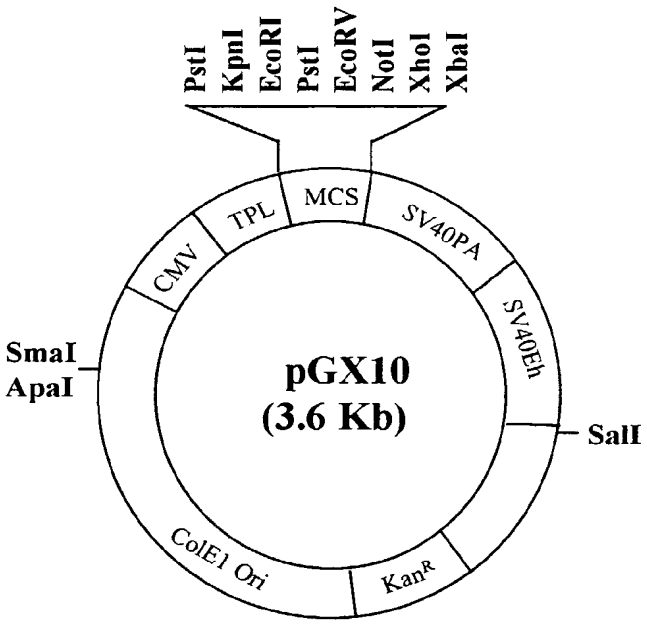

PstI
KpnI
EcoRI
PstI
EcoRV
NotI
XhoI
XbaI
MCS
TPL
CMV
SV40PA
SV40Eh
SmaI
ApaI
pGX10
(3.6 Kb)
SalI
ColE1 Ori
KanR

FIG. 3

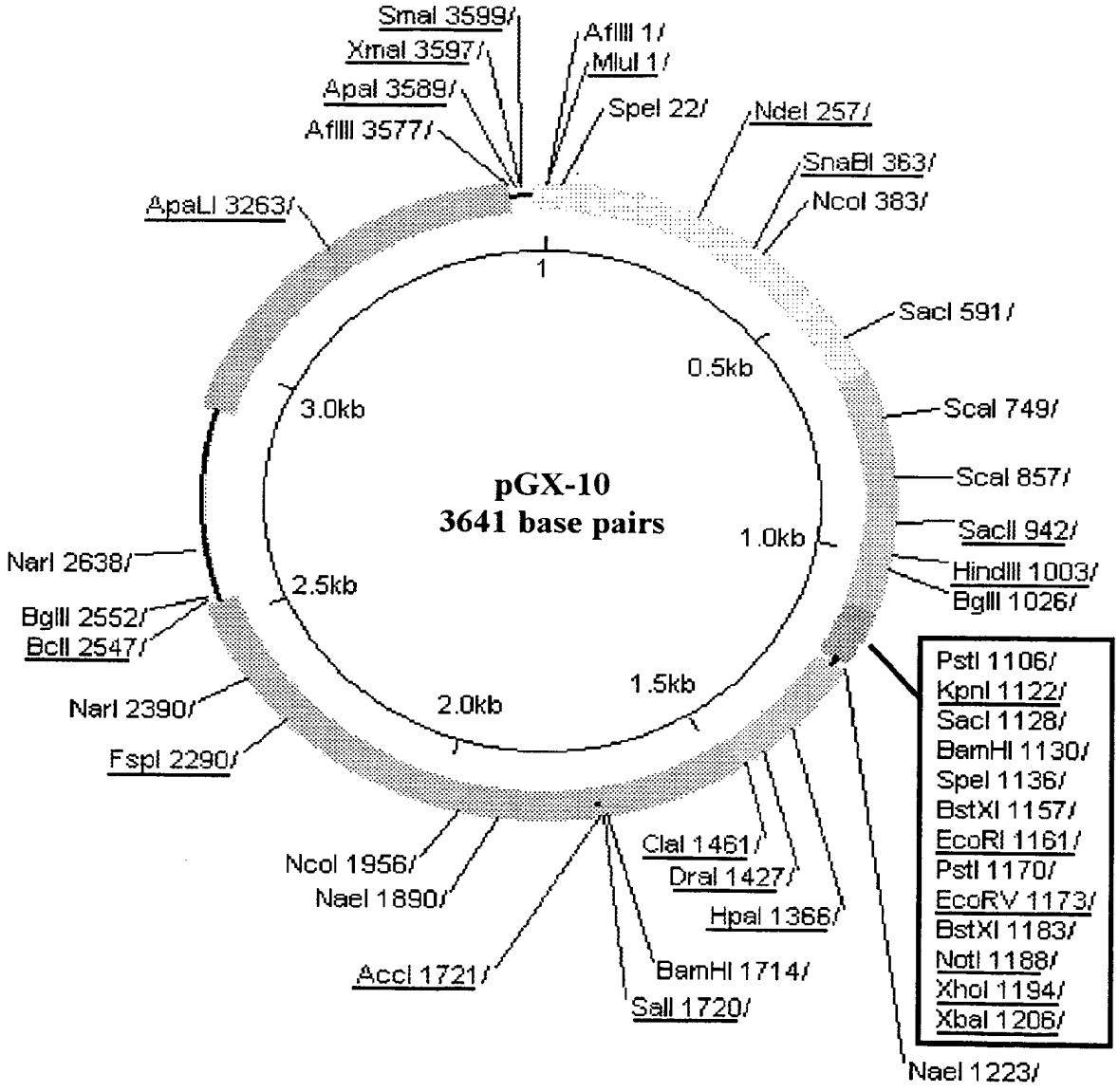

pGX-10
3641 base pairs
1
0.5kb
1.0kb
1.5kb
2.0kb
2.5kb
3.0kb
SmaI 3599/
XmaI 3597/
ApaI 3589/
AflIII 3577/
AflIII 1/
MluI 1/
SpeI 22/
NdeI 257/
SnaBI 363/
NcoI 383/
SacI 591/
ScaI 749/
ScaI 857/
SacII 942/
HindIII 1003/
BglII 1026/
PstI 1106/
KpnI 1122/
SacI 1128/
BamHI 1130/
SpeI 1136/
BstXI 1157/
EcoRI 1161/
PstI 1170/
EcoRV 1173/
BstXI 1183/
NotI 1188/
XhoI 1194/
XbaI 1206/
NaeI 1223/
ClaI 1461/
DraI 1427/
HpaI 1366/
BamHI 1714/
SalI 1720/
AccI 1721/
NcoI 1956/
NaeI 1890/
FspI 2290/
NarI 2390/
BglII 2552/
BclI 2547/
NarI 2638/
ApaLI 3263/

FIG. 4

SIVmac239(template)

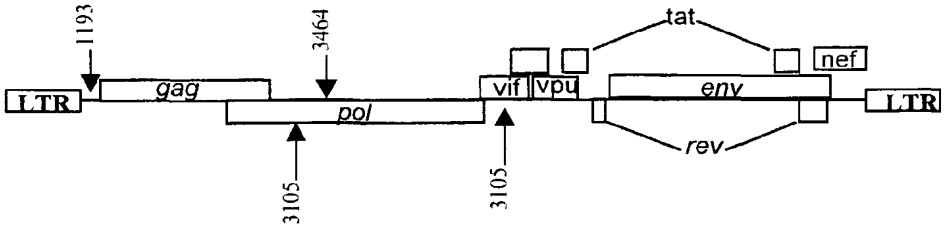


FIG. 5

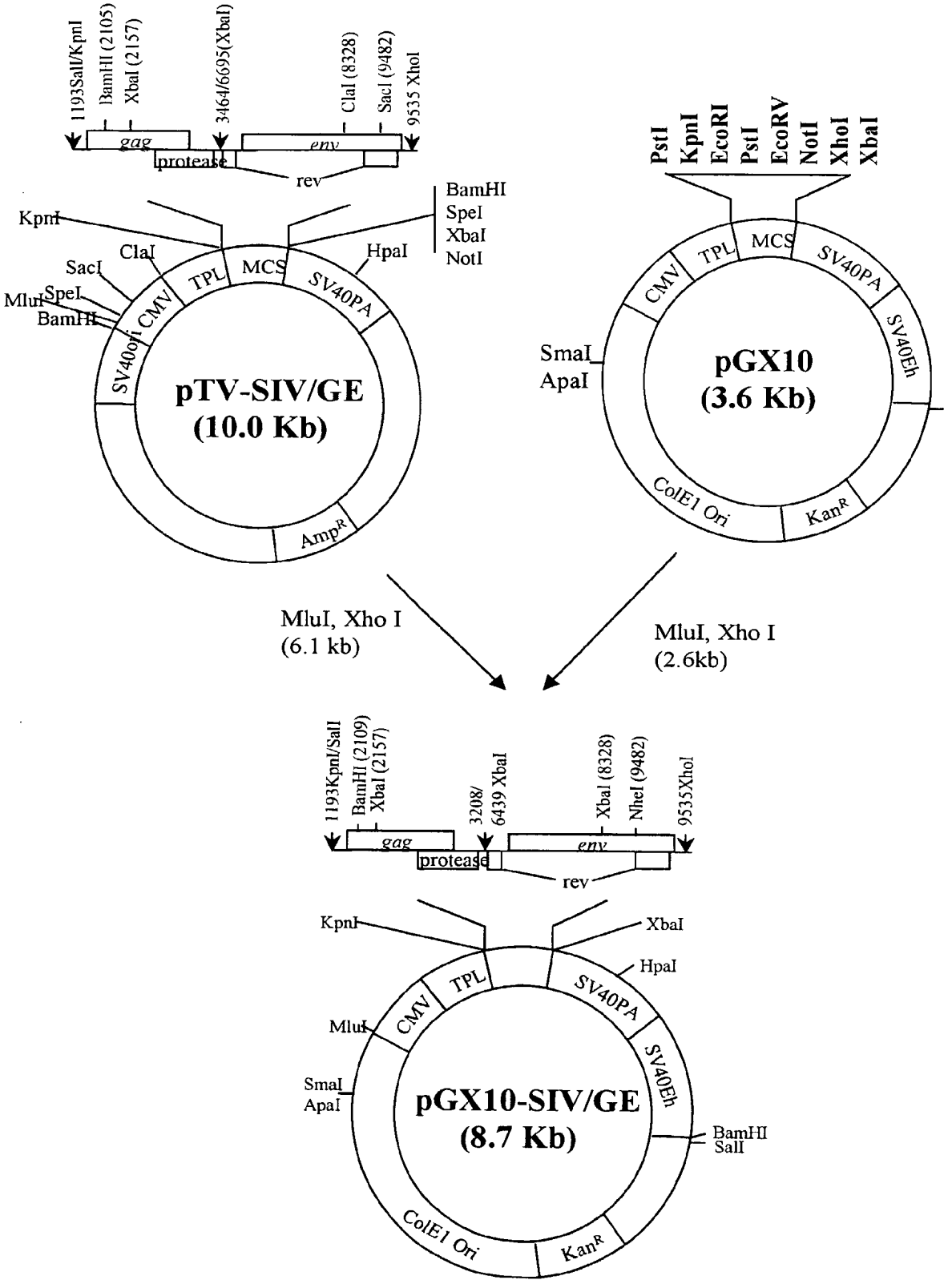

1193SalI/KpnI
BamHI (2105)
XbaI (2157)
3464/6695(XbaI)
ClaI (8328)
SacI (9482)
9535 XhoI
gag
protease
env
rev
KpnI
SacI
ClaI
MluI
SpeI
BamHI
TPL
MCS
CMV
SV40PA
HpaI
BamHI
SpeI
XbaI
NotI
SV40ori
pTV-SIV/GE
(10.0 Kb)
AmpR
PstI
KpnI
EcoRI
PstI
EcoRV
NotI
XhoI
XbaI
SmaI
ApaI
pGX10
(3.6 Kb)
SV40Eh
ColE1 Ori
KanR
MluI, Xho I
(6.1 kb)
MluI, Xho I
(2.6kb)
1193KpnI/SalI
BamHI (2109)
XbaI (2157)
3208/
6439 XbaI
XbaI (8328)
NheI (9482)
9535XhoI
KpnI
XbaI
HpaI
MluI
SmaI
ApaI
pGX10-SIV/GE
(8.7 Kb)
BamHI
SalI

FIG. 6

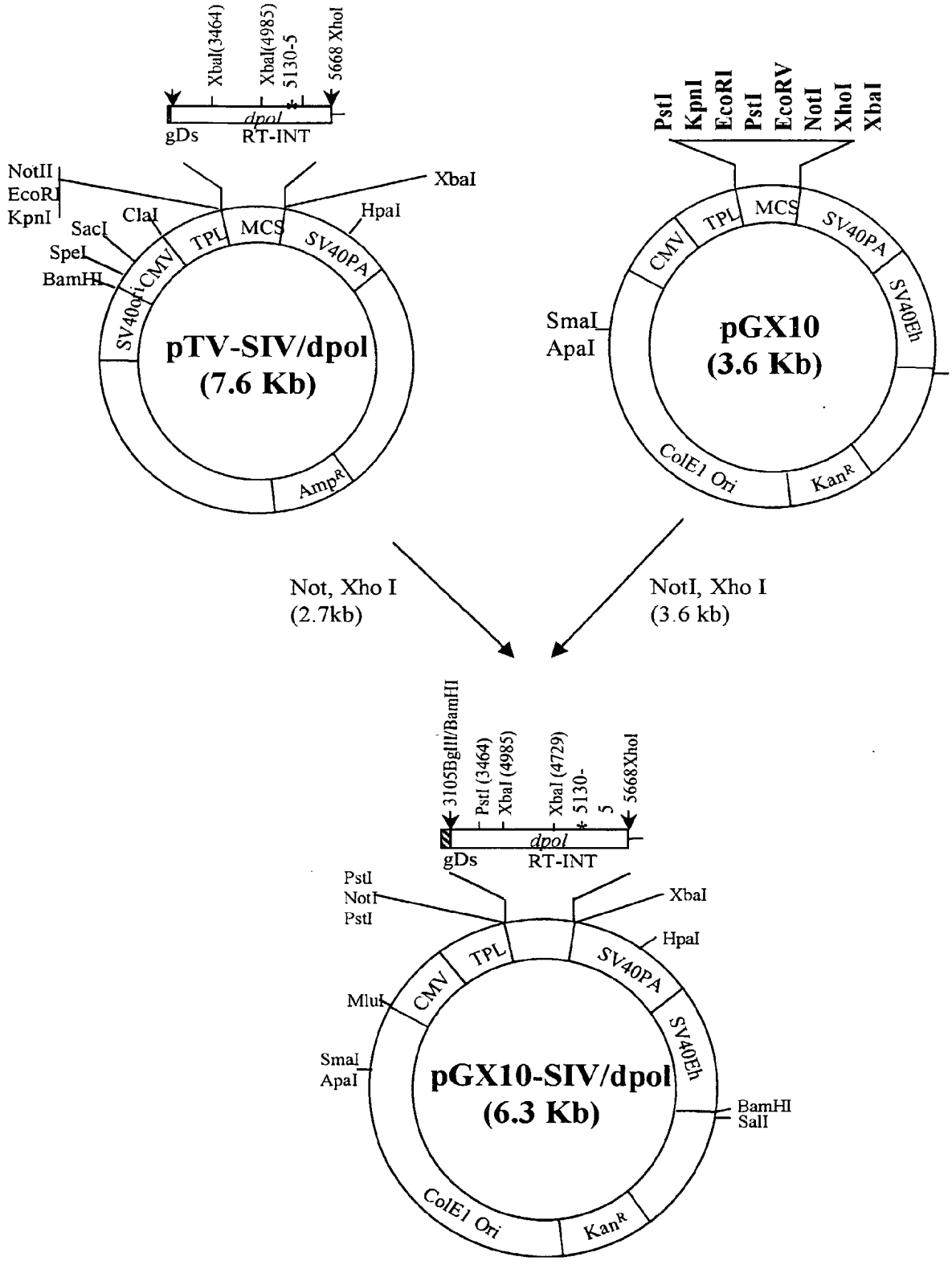

XbaI(3464)
XbaI(4985)
5130-5
5668 XhoI
dpol
gDs
RT-INT
NotII
EcoRI
KpnI
SacI
ClaI
SpeI
BamHI
SV40ori
CMV
TPL
MCS
SV40PA
HpaI
XbaI
pTV-SIV/dpol
(7.6 Kb)
AmpR
PstI
KpnI
EcoRI
PstI
EcoRV
NotI
XhoI
XbaI
SmaI
ApaI
pGX10
(3.6 Kb)
ColE1 Ori
KanR
SV40Eh
Not, Xho I
(2.7kb)
NotI, Xho I
(3.6 kb)
3105BglII/BamHI
PstI (3464)
XbaI (4985)
XbaI (4729)
5130-5
5668XhoI
PstI
NotI
PstI
MluI
pGX10-SIV/dpol
(6.3 Kb)
BamHI
SalI

FIG. 7A

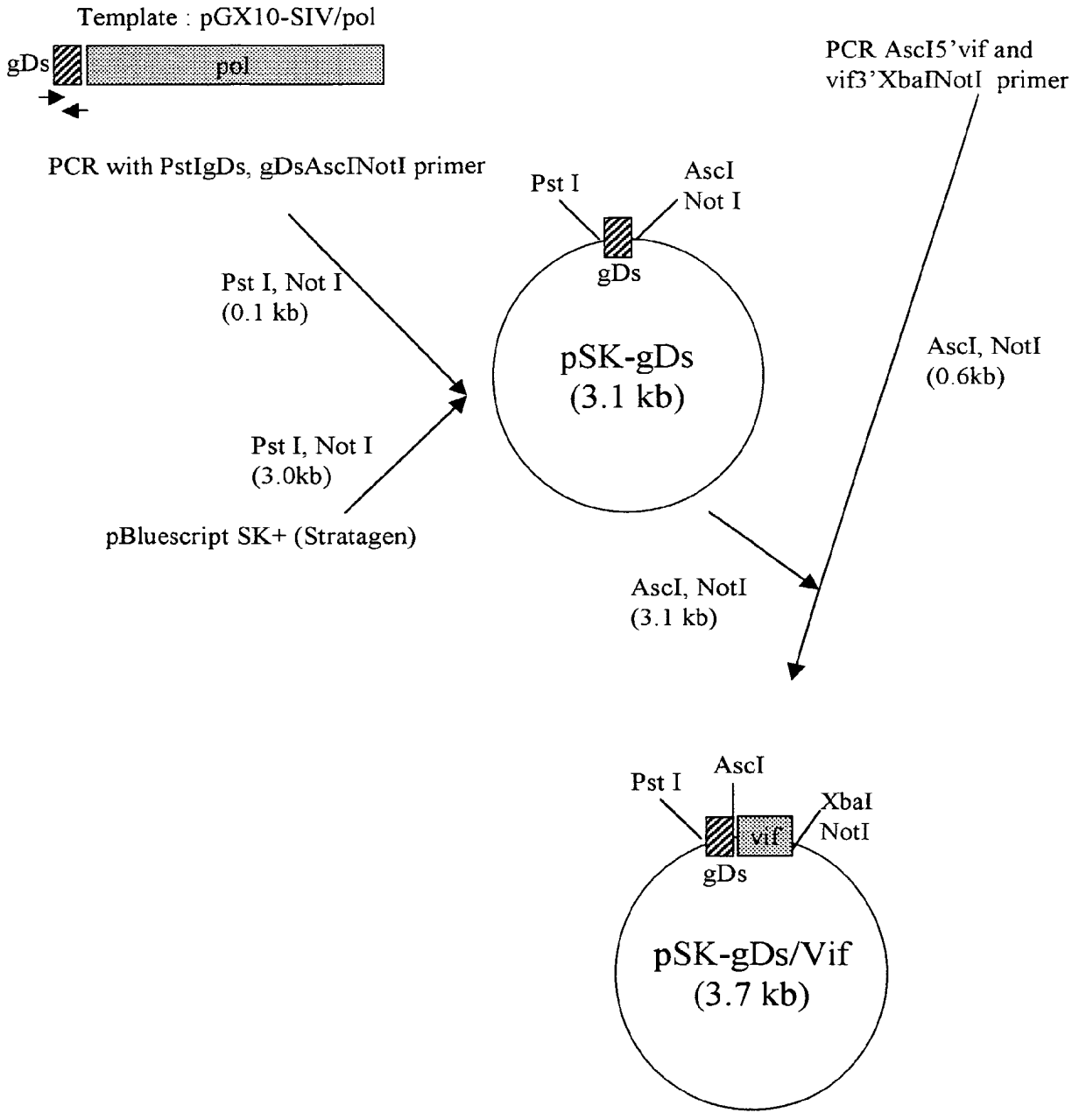

Template : pGX10-SIV/pol
gDs
pol
PCR with PstIgDs, gDsAscINotI primer
PCR AscI5'vif and
vif3'XbaINotI primer
Pst I
AscI
Not I
gDs
Pst I, Not I
(0.1 kb)
pSK-gDs
(3.1 kb)
AscI, NotI
(0.6kb)
Pst I, Not I
(3.0kb)
pBluescript SK+ (Stratagen)
AscI, NotI
(3.1 kb)
Pst I
AscI
XbaI
NotI
vif
gDs
pSK-gDs/Vif
(3.7 kb)

FIG. 7B

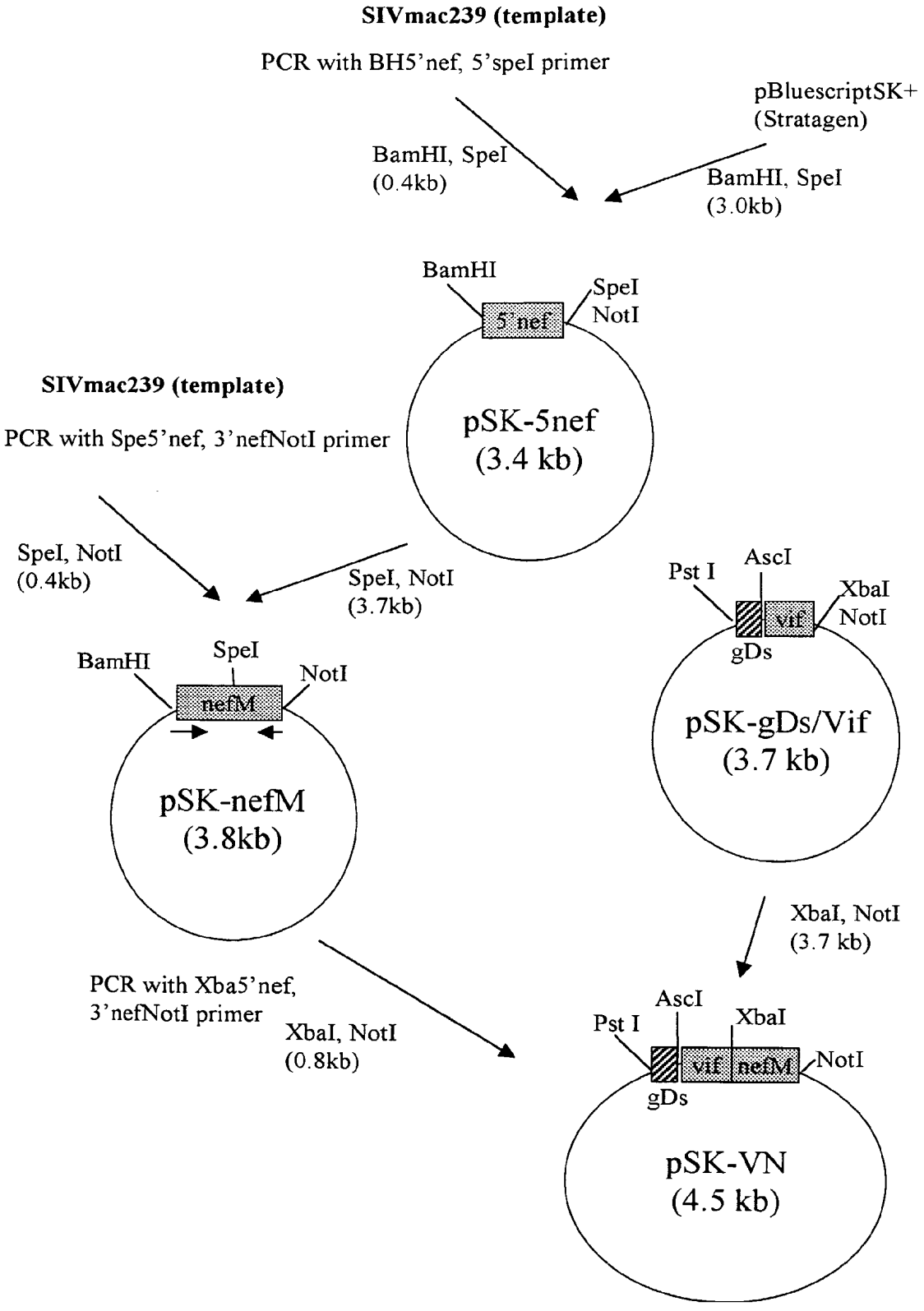

SIVmac239 (template)
PCR with BH5'nef, 5'speI primer
pBluescriptSK+
(Stratagen)
BamHI, SpeI
(0.4kb)
BamHI, SpeI
(3.0kb)
BamHI
SpeI
NotI
5' nef
pSK-5nef
(3.4 kb)
SIVmac239 (template)
PCR with Spe5'nef, 3'nefNotI primer
SpeI, NotI
(0.4kb)
SpeI, NotI
(3.7kb)
BamHI
SpeI
NotI
nefM
pSK-nefM
(3.8kb)
Pst I
AscI
XbaI
NotI
vif
gDs
pSK-gDs/Vif
(3.7 kb)
XbaI, NotI
(3.7 kb)
PCR with Xba5'nef,
3'nefNotI primer
XbaI, NotI
(0.8kb)
AscI
Pst I
XbaI
vif
nefM
NotI
gDs
pSK-VN
(4.5 kb)

FIG. 7C

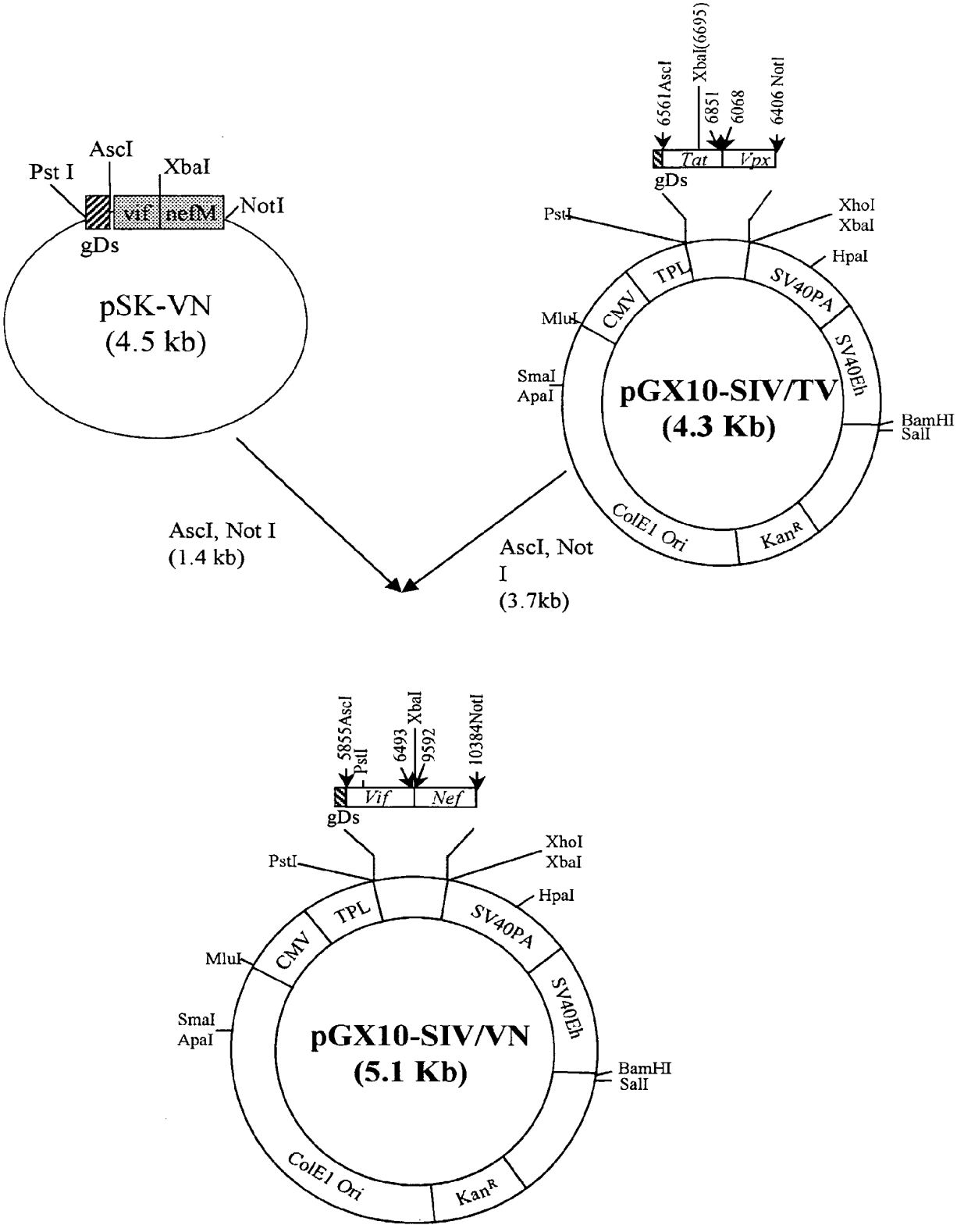

AscI
Pst I
XbaI
vif
nefM
NotI
gDs
pSK-VN
(4.5 kb)
AscI, Not I
(1.4 kb)
6561AscI
XbaI(6695)
6851
6068
6406 NotI
Tat
Vpx
gDs
PstI
XhoI
XbaI
HpaI
TPL
CMV
SV40PA
SV40Eh
MluI
SmaI
ApaI
pGX10-SIV/TV
(4.3 Kb)
BamHI
SalI
ColE1 Ori
KanR
AscI, Not
I
(3.7kb)
5855AscI
PstI
6493
XbaI
9592
10384NotI
Vif
Nef
gDs
PstI
XhoI
XbaI
HpaI
TPL
CMV
SV40PA
SV40Eh
MluI
SmaI
ApaI
pGX10-SIV/VN
(5.1 Kb)
BamHI
SalI
ColE1 Ori
KanR

FIG. 8A

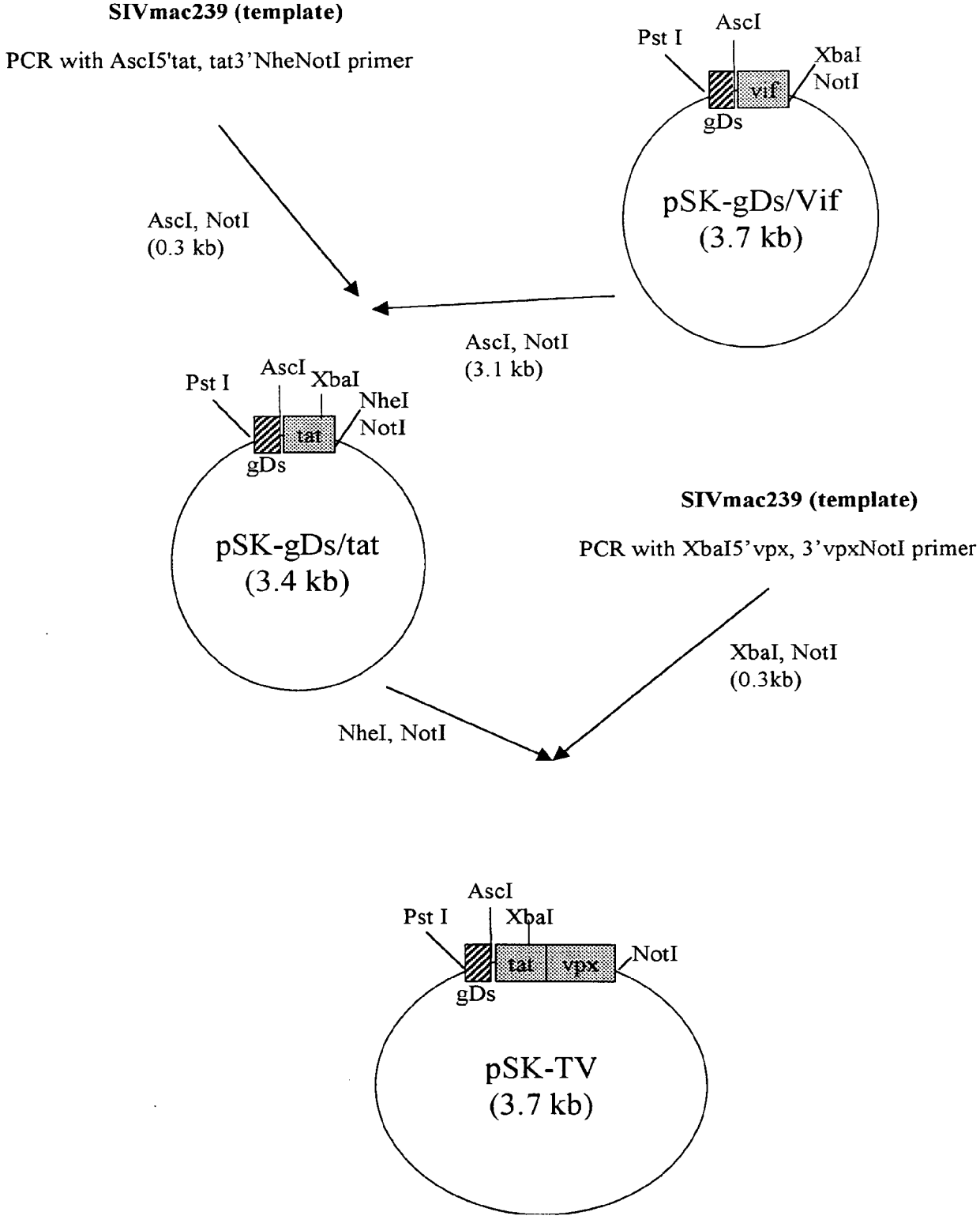

SIVmac239 (template)
PCR with AscI5'tat, tat3'NheNotI primer
Pst I
AscI
XbaI
NotI
vif
gDs
pSK-gDs/Vif
(3.7 kb)
AscI, NotI
(0.3 kb)
AscI, NotI
(3.1 kb)
Pst I
AscI
XbaI
NheI
NotI
tat
gDs
pSK-gDs/tat
(3.4 kb)
SIVmac239 (template)
PCR with XbaI5'vpx, 3'vpxNotI primer
XbaI, NotI
(0.3kb)
NheI, NotI
AscI
Pst I
XbaI
tat
vpx
NotI
gDs
pSK-TV
(3.7 kb)

FIG. 8B

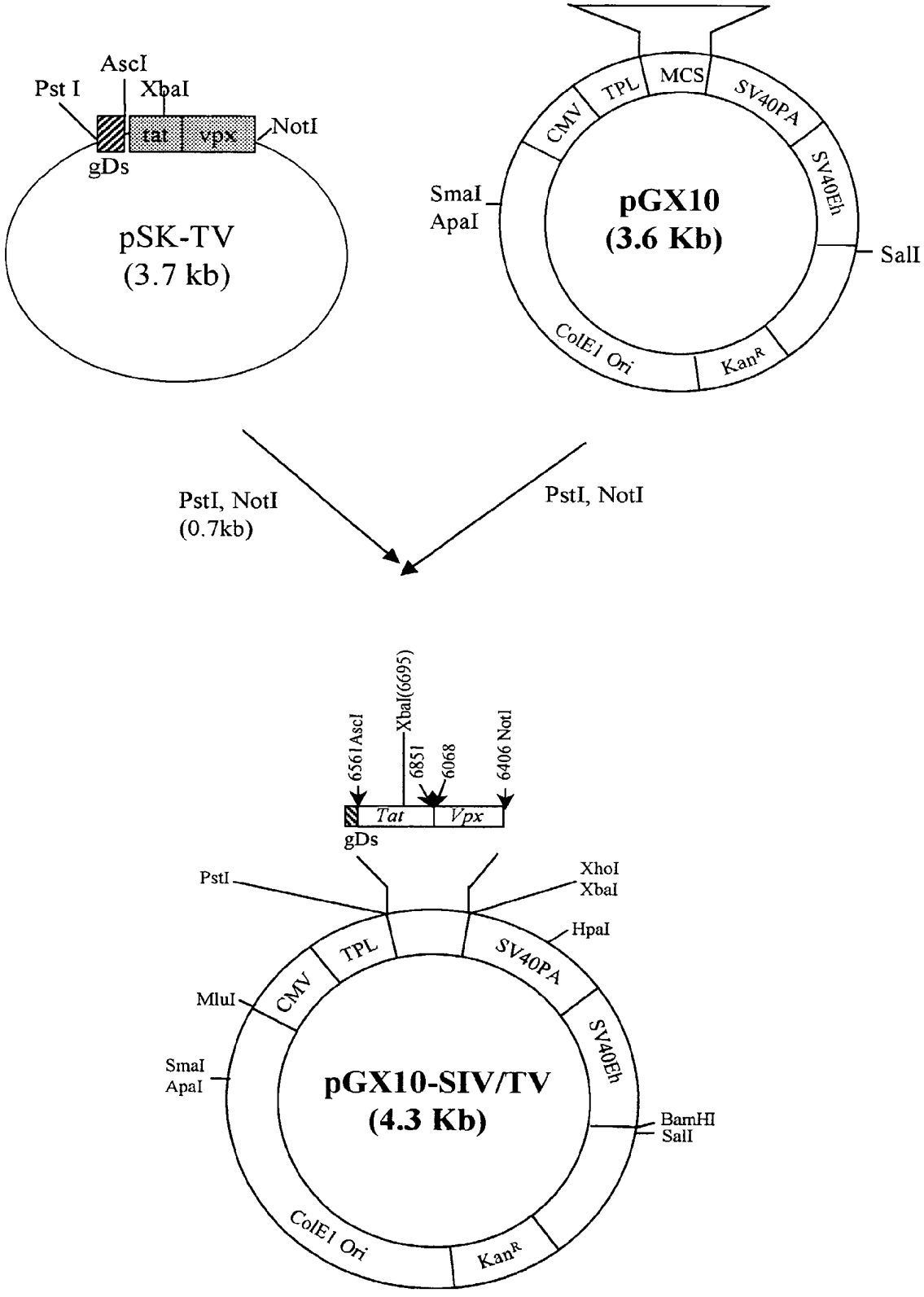

AscI
Pst I
XbaI
tat
vpx
NotI
gDs
pSK-TV
(3.7 kb)
MCS
TPL
SV40PA
CMV
SV40Eh
SmaI
ApaI
pGX10
(3.6 Kb)
SalI
ColE1 Ori
Kan^R
PstI, NotI
(0.7kb)
PstI, NotI
6361AscI
XbaI(6695)
6851
6068
6406 NotI
Tat
Vpx
gDs
PstI
XhoI
XbaI
HpaI
TPL
SV40PA
MluI
CMV
SV40Eh
SmaI
ApaI
pGX10-SIV/TV
(4.3 Kb)
BamHI
SalI
ColE1 Ori
Kan^R

FIG. 9

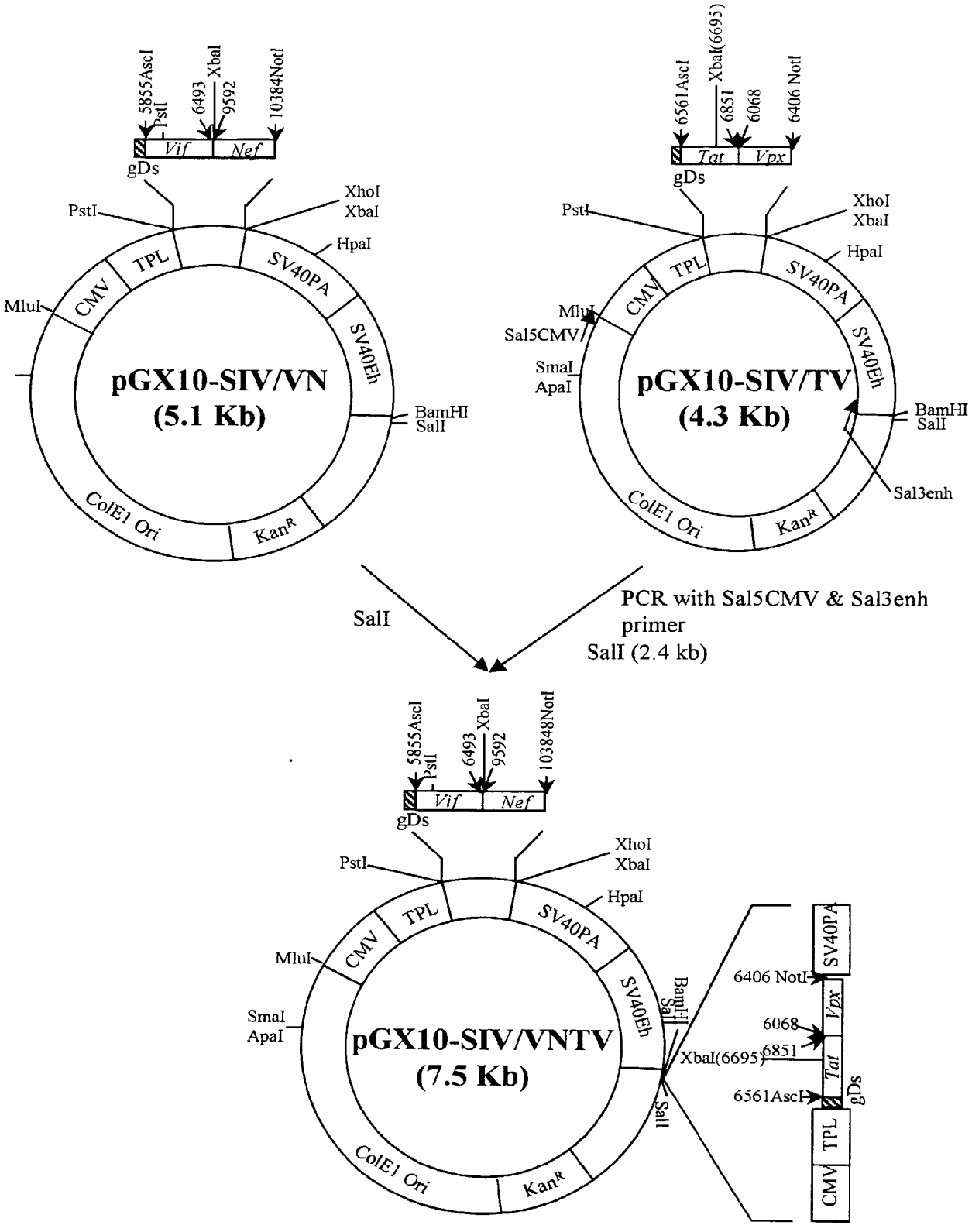

5855AscI
PstI
6493
XbaI
9592
10384NotI
Vif
Nef
gDs
PstI
XhoI
XbaI
HpaI
MluI
CMV
TPL
SV40PA
SV40Eh
pGX10-SIV/VN
(5.1 Kb)
BamHI
SalI
ColE1 Ori
KanR
6561AscI
XbaI(6695)
6851
6068
6406 NotI
Tat
Vpx
Sal5CMV
SmaI
ApaI
pGX10-SIV/TV
(4.3 Kb)
Sal3enh
SalI
PCR with Sal5CMV & Sal3enh primer
SalI (2.4 kb)
103848NotI
pGX10-SIV/VNTV
(7.5 Kb)

FIG. 10

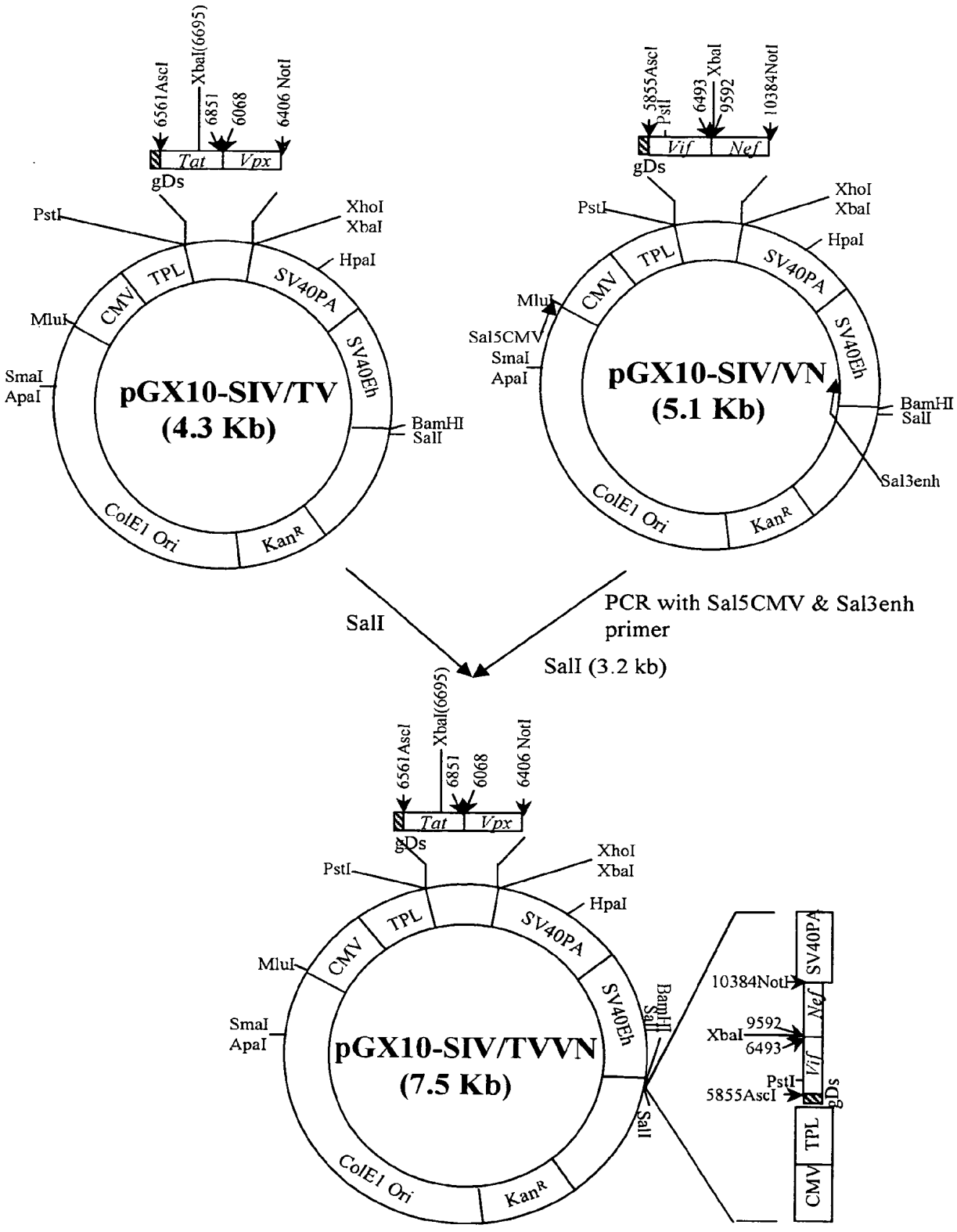

6561AscI
XbaI(6695)
6851
6068
6406 NotI
Tat
Vpx
gDs
PstI
XhoI
XbaI
HpaI
SV40PA
SV40Eh
MluI
CMV
TPL
SmaI
ApaI
pGX10-SIV/TV
(4.3 Kb)
BamHI
SalI
ColE1 Ori
KanR
5855AscI
PstI
6493
9592
10384NotI
Vif
Nef
Sal5CMV
pGX10-SIV/VN
(5.1 Kb)
Sal3enh
SalI
PCR with Sal5CMV & Sal3enh primer
SalI (3.2 kb)
pGX10-SIV/TVVN
(7.5 Kb)
10384NotI
9592
XbaI
6493
5855AscI

FIG. 11

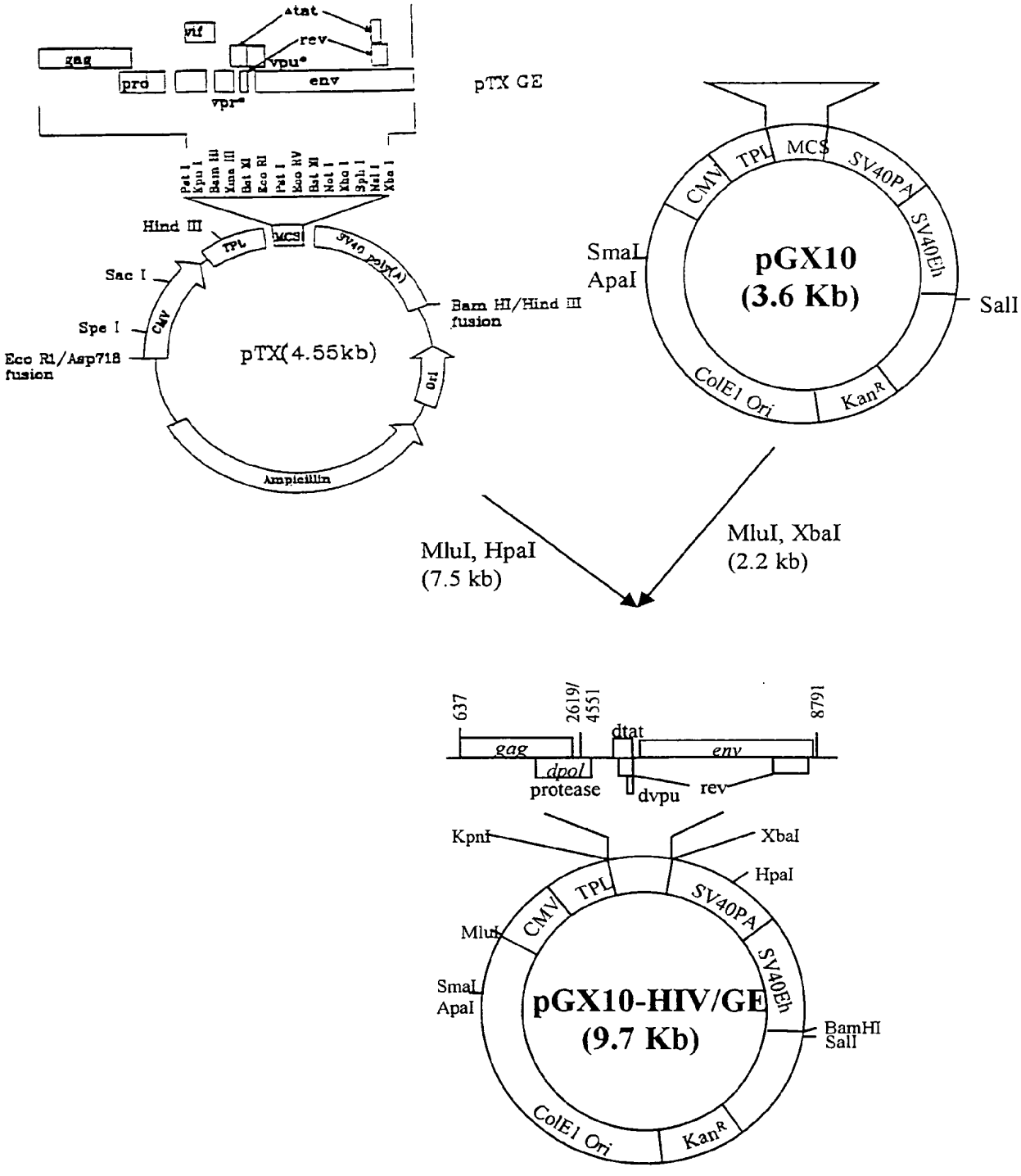

gag
pro
vif
vpr
tat
rev
vpu
env
pTX GE
Hind III
Sac I
Spe I
Eco RI/Asp718 fusion
Bam HI/Hind III fusion
TPL
MCS
SV40 poly(A)
CMV
ori
Ampicillin
pTX(4.55kb)
SmaI
ApaI
SalI
CMV
TPL
MCS
SV40PA
SV40Eh
ColE1 Ori
KanR
pGX10
(3.6 Kb)
MluI, HpaI
(7.5 kb)
MluI, XbaI
(2.2 kb)
637
2619/
4551
8791
gag
dpol
protease
dtat
dvpu
rev
env
KpnI
XbaI
HpaI
MluI
SmaI
ApaI
BamHI
SalI
pGX10-HIV/GE
(9.7 Kb)

FIG. 12A

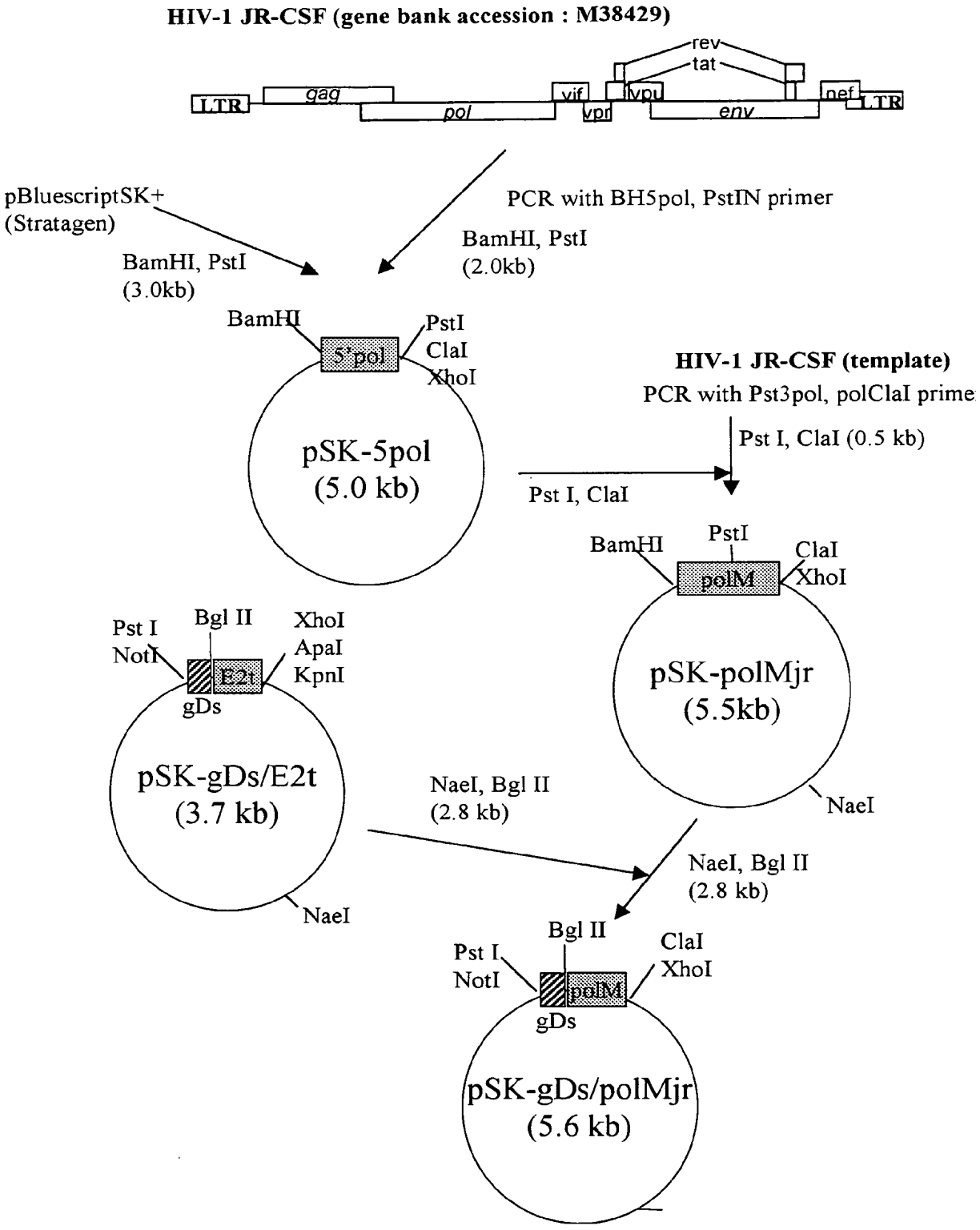

HIV-1 JR-CSF (gene bank accession : M38429)
rev
tat
LTR
gag
pol
vif
vpr
vpu
env
nef
LTR
pBluescriptSK+
(Stratagen)
BamHI, PstI
(3.0kb)
PCR with BH5pol, PstIN primer
BamHI, PstI
(2.0kb)
BamHI
PstI
ClaI
XhoI
5'pol
pSK-5pol
(5.0 kb)
HIV-1 JR-CSF (template)
PCR with Pst3pol, polClaI prime
Pst I, ClaI (0.5 kb)
Pst I, ClaI
BamHI
PstI
ClaI
XhoI
polM
pSK-polMjr
(5.5kb)
NaeI
Pst I
NotI
Bgl II
XhoI
ApaI
KpnI
E2t
gDs
pSK-gDs/E2t
(3.7 kb)
NaeI
NaeI, Bgl II
(2.8 kb)
NaeI, Bgl II
(2.8 kb)
Bgl II
Pst I
NotI
ClaI
XhoI
polM
gDs
pSK-gDs/polMjr
(5.6 kb)

FIG. 12B

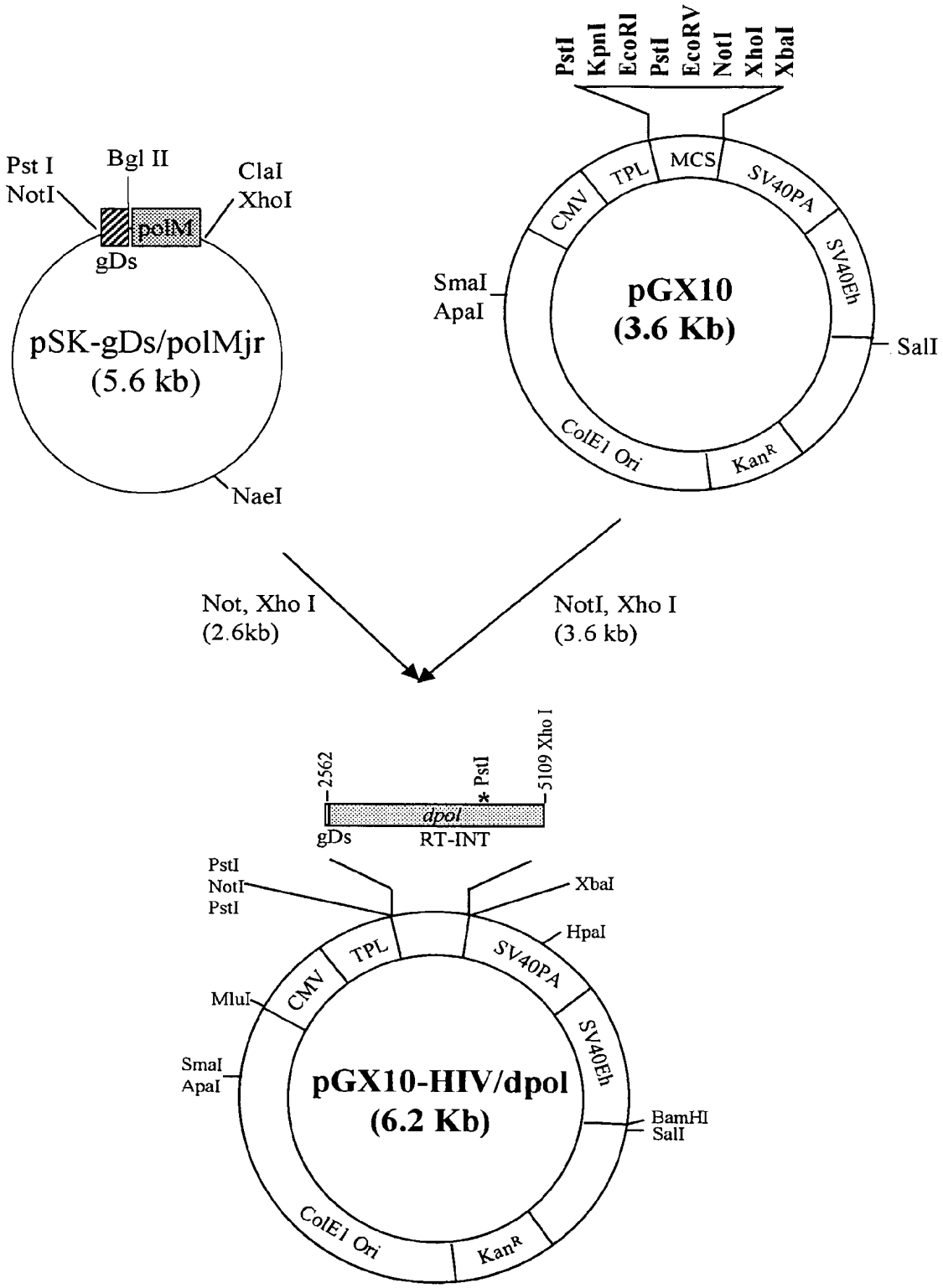

PstI
KpnI
EcoRI
PstI
EcoRV
NotI
XhoI
XbaI
Pst I
NotI
Bgl II
ClaI
XhoI
polM
gDs
pSK-gDs/polMjr
(5.6 kb)
NaeI
MCS
TPL
CMV
SV40PA
SV40Eh
SmaI
ApaI
pGX10
(3.6 Kb)
SalI
ColE1 Ori
KanR
Not, Xho I
(2.6kb)
NotI, Xho I
(3.6 kb)
2562
PstI
5109 Xho I
dpol
gDs
RT-INT
PstI
NotI
PstI
XbaI
HpaI
TPL
CMV
SV40PA
SV40Eh
MluI
SmaI
ApaI
pGX10-HIV/dpol
(6.2 Kb)
BamHI
SalI
ColE1 Ori
KanR

FIG. 13A

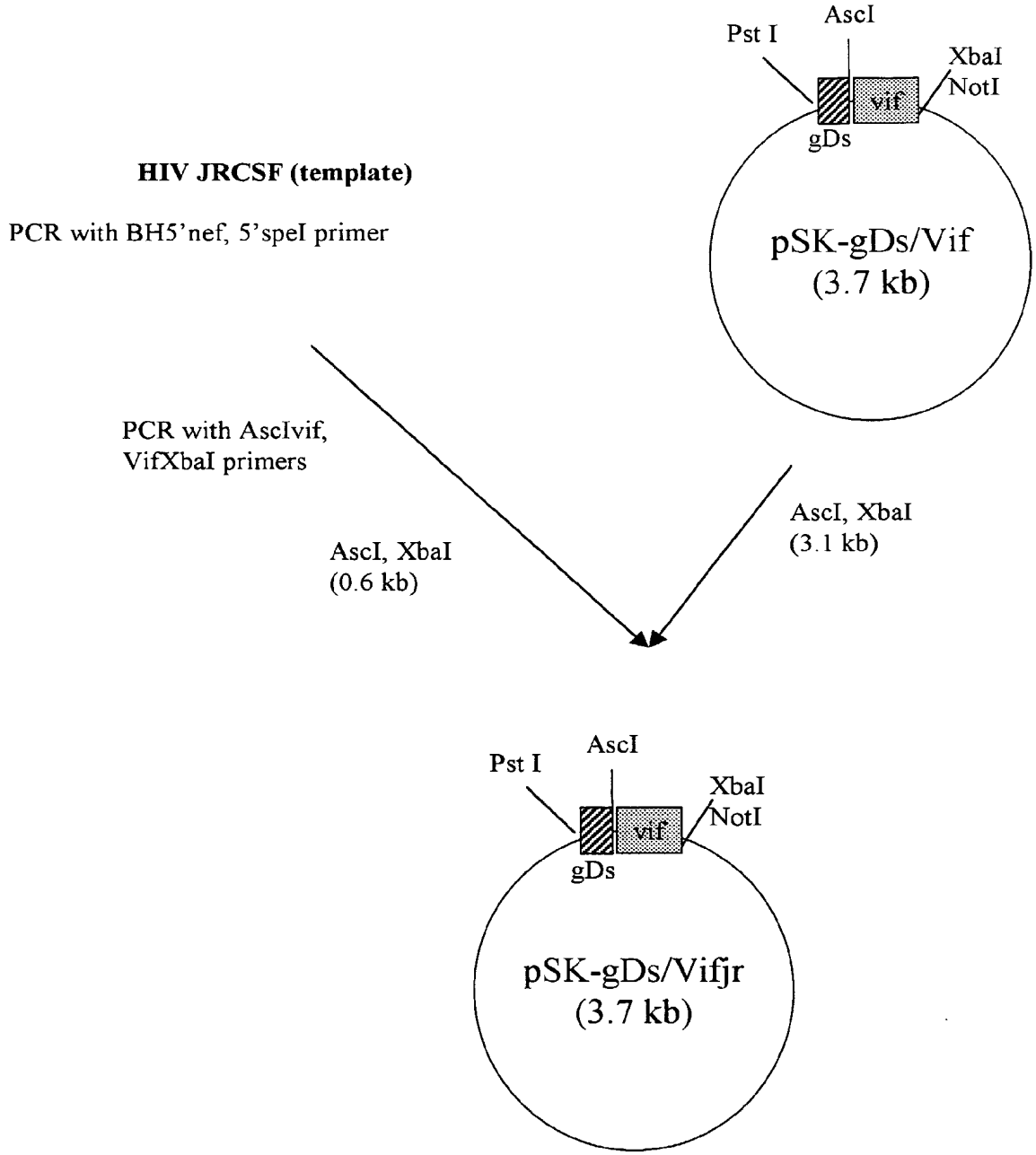

Pst I
AscI
XbaI
NotI
vif
gDs
pSK-gDs/Vif
(3.7 kb)
HIV JRCSF (template)
PCR with BH5'nef, 5'speI primer
PCR with AscIvif,
VifXbaI primers
AscI, XbaI
(0.6 kb)
AscI, XbaI
(3.1 kb)
Pst I
AscI
XbaI
NotI
vif
gDs
pSK-gDs/Vifjr
(3.7 kb)

FIG. 13B

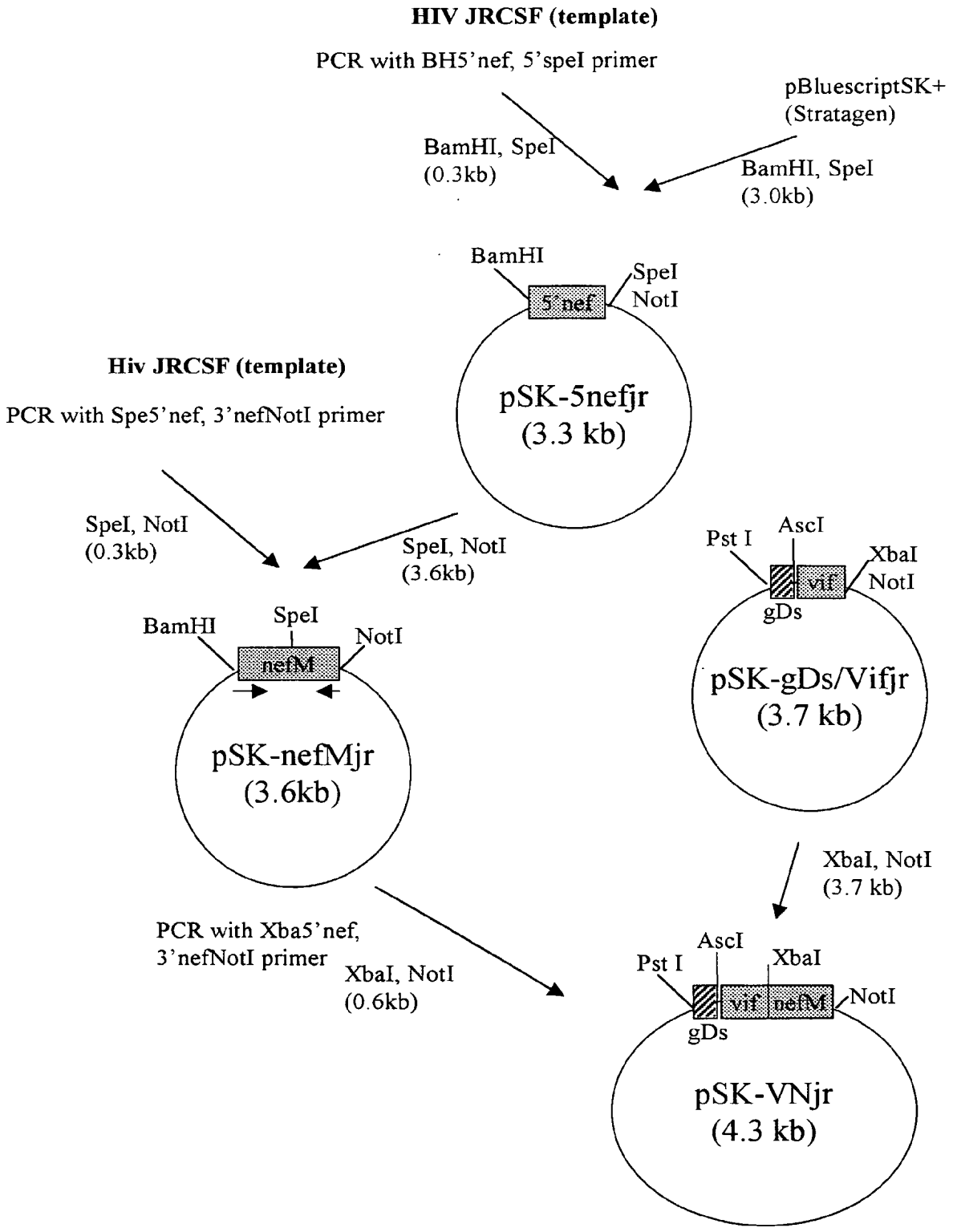

HIV JRCSF (template)
PCR with BH5'nef, 5'speI primer
pBluescriptSK+
(Stratagen)
BamHI, SpeI
(0.3kb)
BamHI, SpeI
(3.0kb)
BamHI
SpeI
NotI
5'nef
pSK-5nefjr
(3.3 kb)
Hiv JRCSF (template)
PCR with Spe5'nef, 3'nefNotI primer
SpeI, NotI
(0.3kb)
SpeI, NotI
(3.6kb)
BamHI
SpeI
NotI
nefM
pSK-nefMjr
(3.6kb)
Pst I
AscI
XbaI
NotI
vif
gDs
pSK-gDs/Vifjr
(3.7 kb)
XbaI, NotI
(3.7 kb)
PCR with Xba5'nef,
3'nefNotI primer
XbaI, NotI
(0.6kb)
Pst I
AscI
XbaI
NotI
vif
nefM
gDs
pSK-VNjr
(4.3 kb)

FIG. 13C

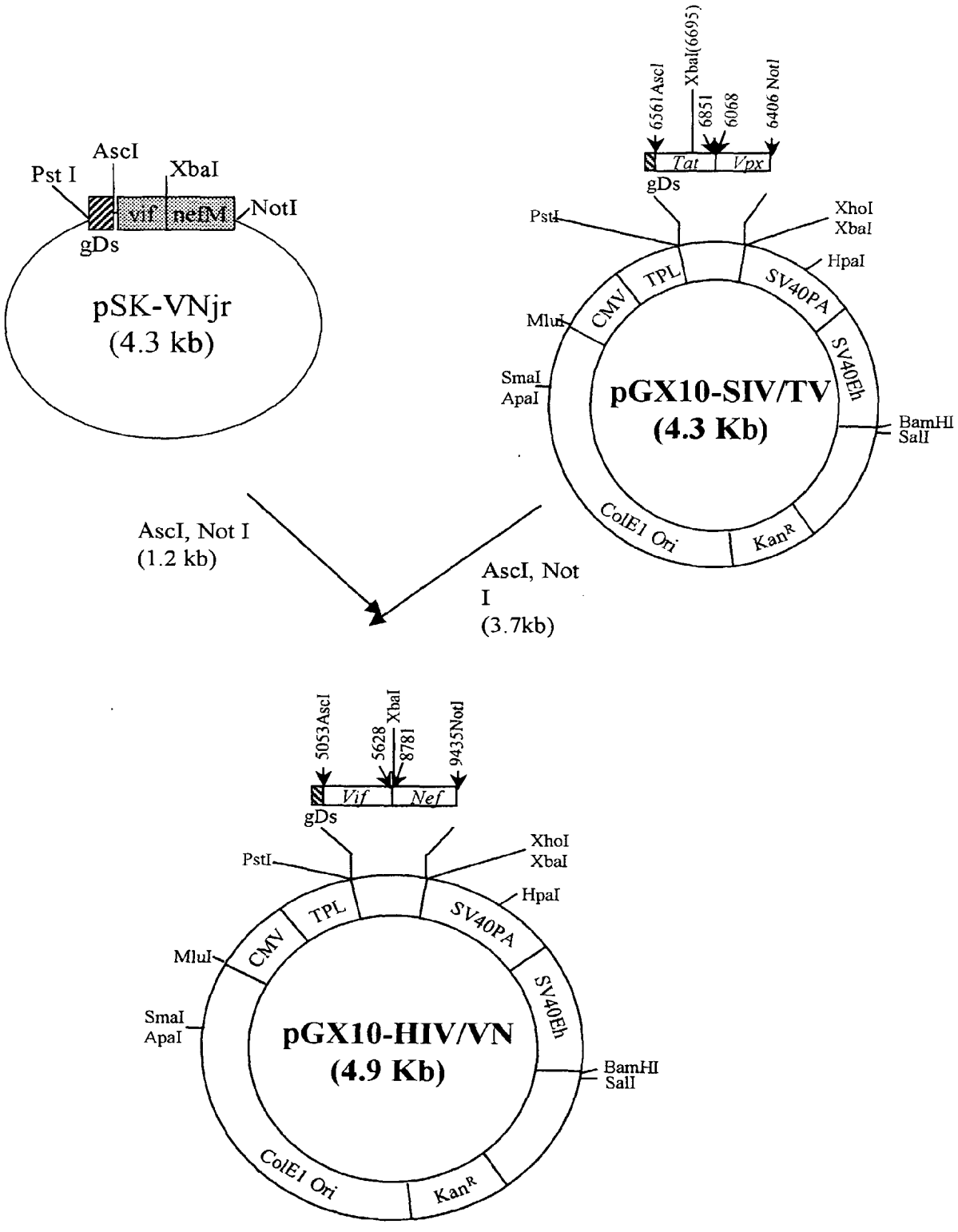

AscI
Pst I
XbaI
vif
nefM
NotI
gDs
pSK-VNjr
(4.3 kb)
AscI, Not I
(1.2 kb)
AscI, Not
I
(3.7kb)
6361AscI
XbaI(6695)
6851
6068
6406 NotI
Tat
Vpx
gDs
PstI
XhoI
XbaI
HpaI
TPL
SV40PA
CMV
MluI
SV40Eh
SmaI
ApaI
pGX10-SIV/TV
(4.3 Kb)
BamHI
SalI
ColE1 Ori
KanR
5053AscI
XbaI
5628
8781
9435NotI
Vif
Nef
gDs
PstI
XhoI
XbaI
HpaI
TPL
SV40PA
CMV
MluI
SV40Eh
SmaI
ApaI
pGX10-HIV/VN
(4.9 Kb)
BamHI
SalI
ColE1 Ori
KanR

FIG. 14A

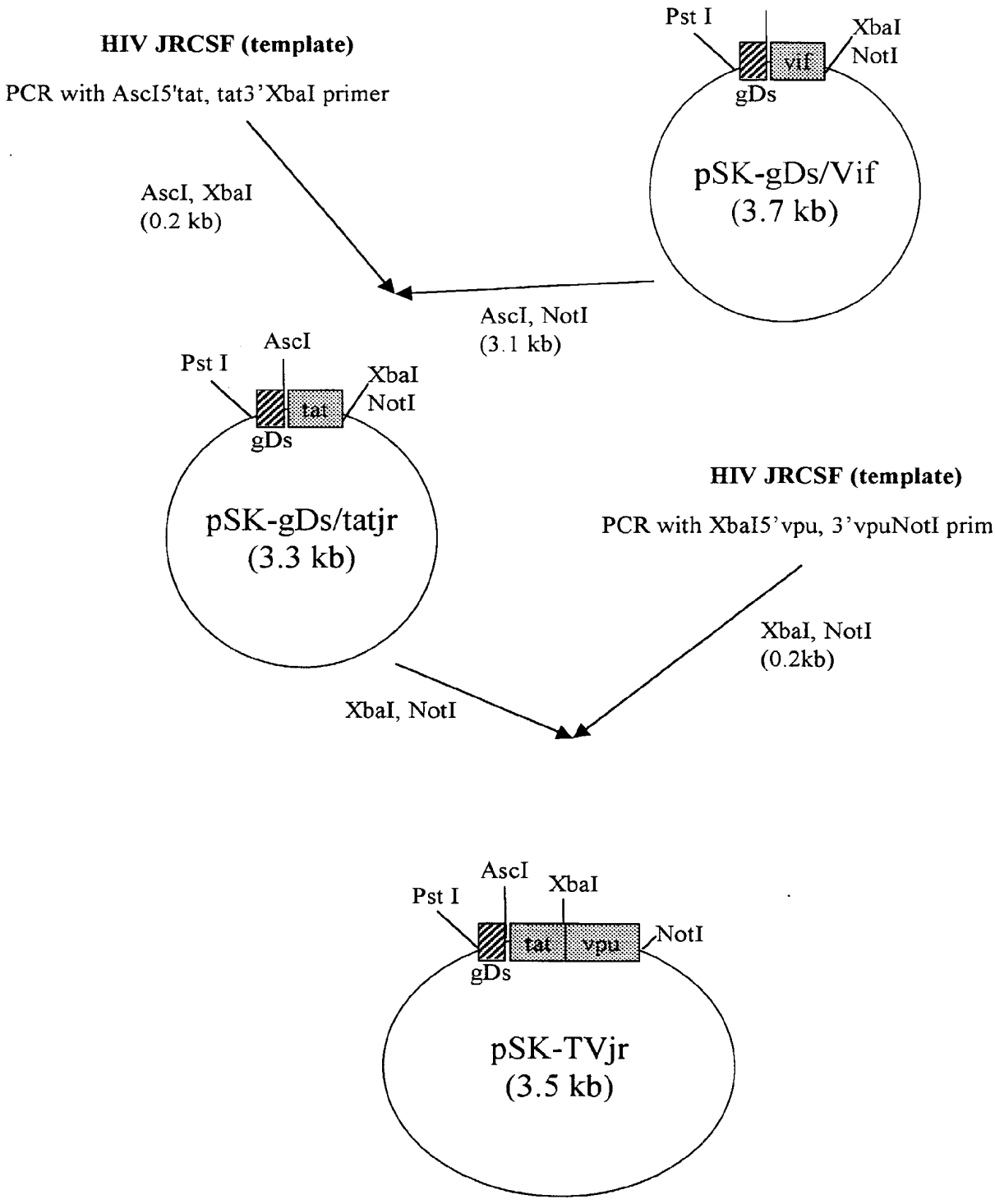

HIV JRCSF (template)
PCR with AscI5'tat, tat3'XbaI primer
Pst I
XbaI
NotI
vif
gDs
pSK-gDs/Vif
(3.7 kb)
AscI, XbaI
(0.2 kb)
AscI, NotI
(3.1 kb)
AscI
Pst I
XbaI
NotI
tat
gDs
pSK-gDs/tatjr
(3.3 kb)
HIV JRCSF (template)
PCR with XbaI5'vpu, 3'vpuNotI prim
XbaI, NotI
(0.2kb)
XbaI, NotI
AscI
Pst I
XbaI
tat
vpu
NotI
gDs
pSK-TVjr
(3.5 kb)

FIG. 14B

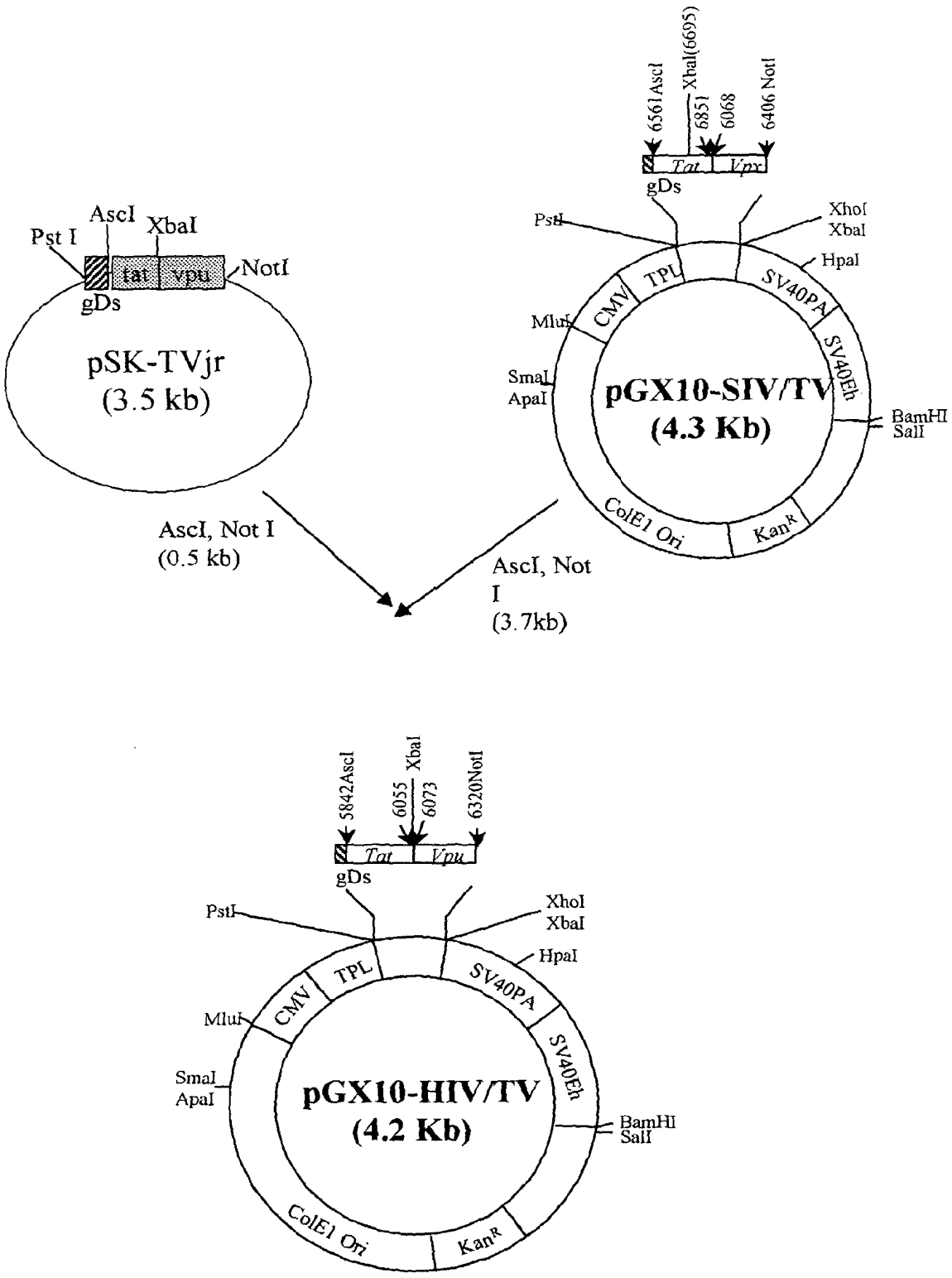

pSK-TVjr
(3.5 kb)
Pst I
AscI
XbaI
tat
vpu
NotI
gDs
AscI, Not I
(0.5 kb)
AscI, Not I
(3.7kb)
pGX10-SIV/TV
(4.3 Kb)
6561AscI
XbaI(6695)
6851
6068
6406 NotI
Tat
Vpx
gDs
PstI
XhoI
XbaI
HpaI
TPL
CMV
SV40PA
SV40Eh
MluI
SmaI
ApaI
BamHI
SalI
ColE1 Ori
Kan^R
pGX10-HIV/TV
(4.2 Kb)
5842AscI
XbaI
6055
6073
6320NotI
Tat
Vpu
gDs
PstI
XhoI
XbaI
HpaI
TPL
CMV
SV40PA
SV40Eh
MluI
SmaI
ApaI
BamHI
SalI
ColE1 Ori
Kan^R

FIG. 15

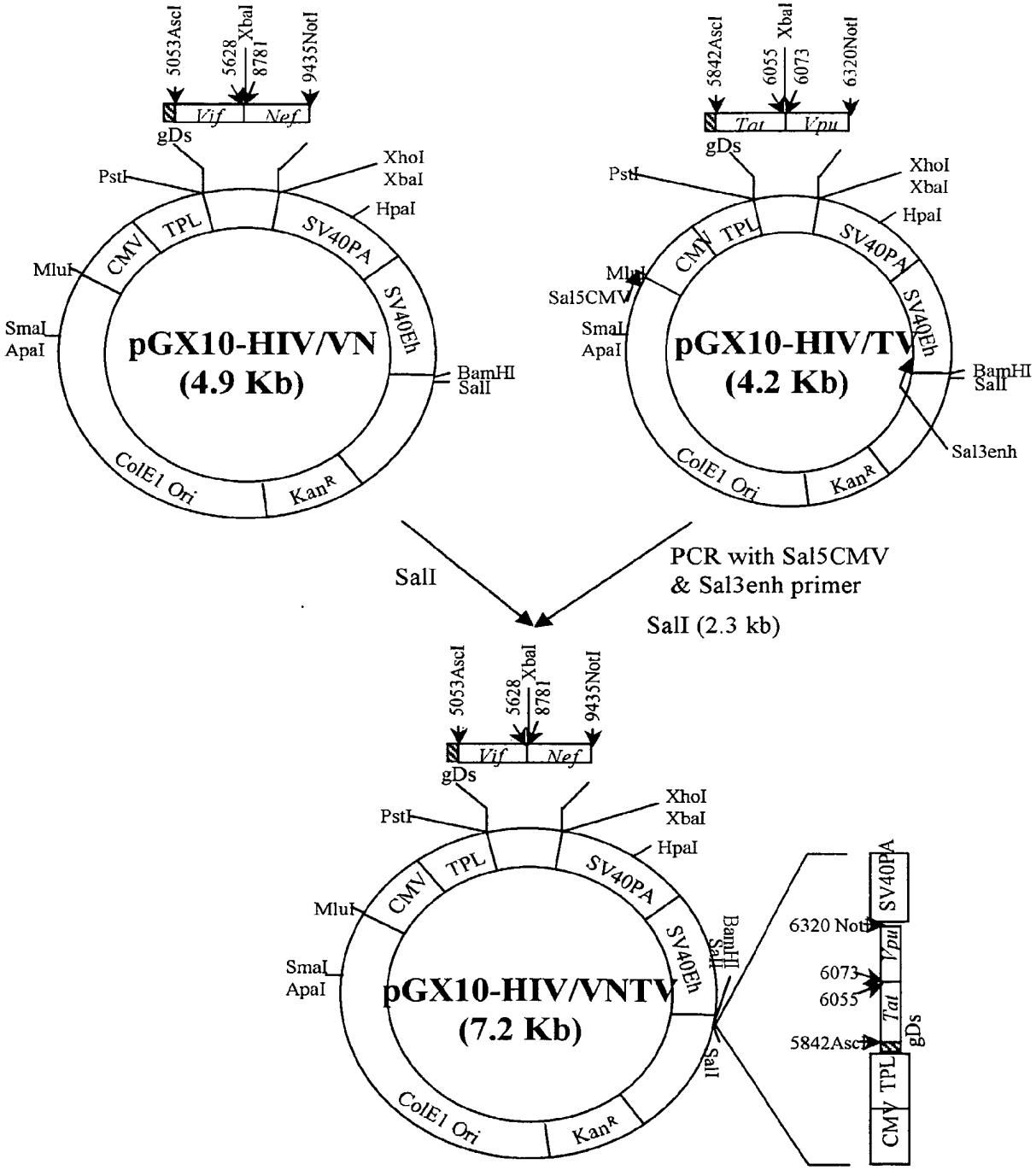

5053AscI
5628
XbaI
8781
9435NotI
Vif
Nef
gDs
PstI
XhoI
XbaI
HpaI
SV40PA
SV40Eh
MluI
CMV
TPL
SmaI
ApaI
pGX10-HIV/VN
(4.9 Kb)
BamHI
SalI
ColE1 Ori
KanR
5842AscI
6055
6073
6320NotI
Tat
Vpu
Sal5CMV
Sal3enh
pGX10-HIV/TV
(4.2 Kb)
SalI
PCR with Sal5CMV
& Sal3enh primer
SalI (2.3 kb)
pGX10-HIV/VNTV
(7.2 Kb)
6320 NotI
6073
6055
5842AscI

FIG. 16

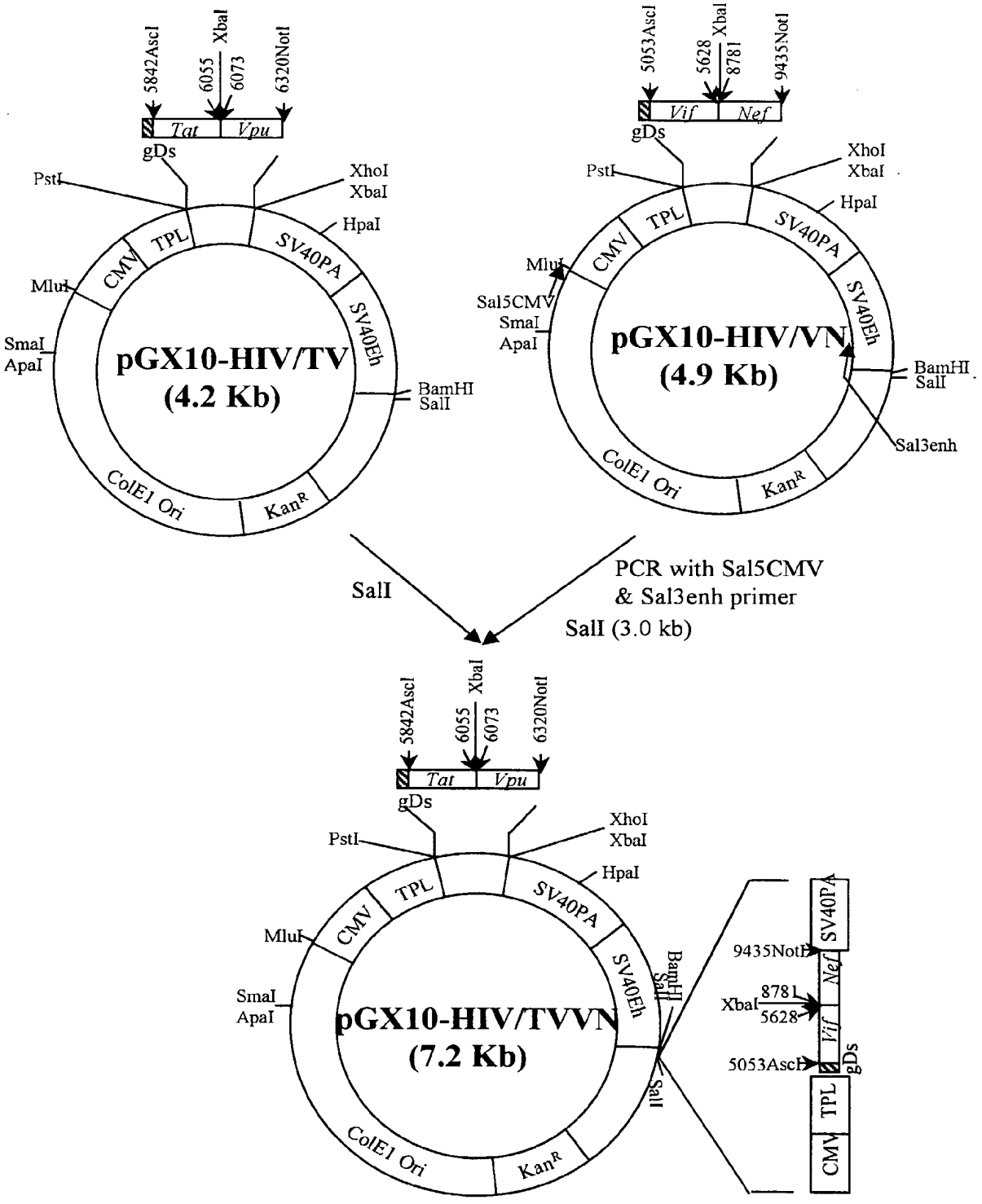

5842AscI
6055
XbaI
6073
6320NotI
Tat
Vpu
gDs
PstI
XhoI
XbaI
HpaI
SV40PA
SV40Eh
BamHI
SalI
ColE1 Ori
KanR
CMV
TPL
MluI
SmaI
ApaI
pGX10-HIV/TV
(4.2 Kb)
5053AscI
5628
8781
9435NotI
Vif
Nef
Sal5CMV
Sal3enh
pGX10-HIV/VN
(4.9 Kb)
SalI
PCR with Sal5CMV
& Sal3enh primer
SalI (3.0 kb)
pGX10-HIV/TVVN
(7.2 Kb)

FIG. 17A

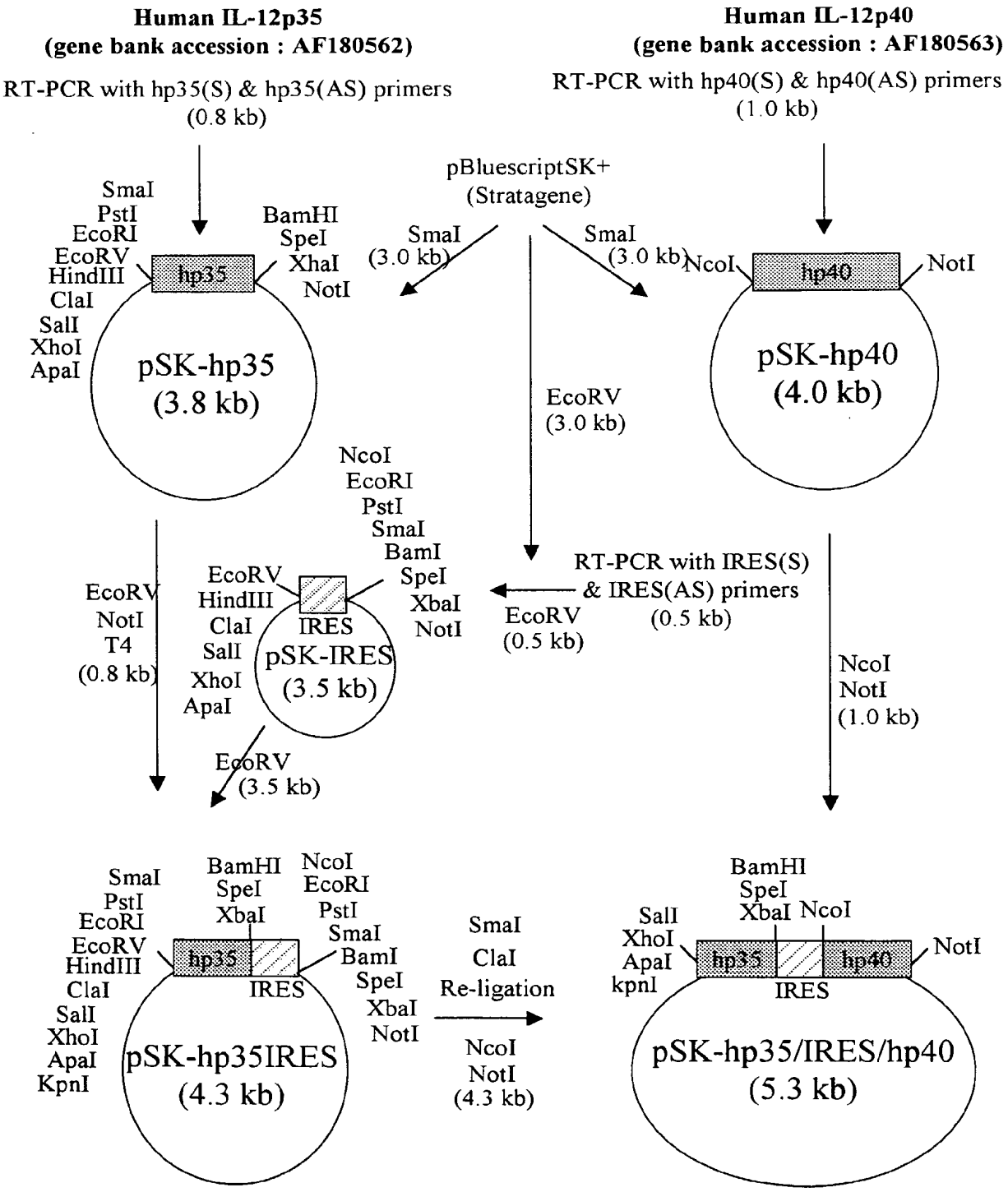

Human IL-12p35
(gene bank accession : AF180562)
RT-PCR with hp35(S) & hp35(AS) primers
(0.8 kb)
Human IL-12p40
(gene bank accession : AF180563)
RT-PCR with hp40(S) & hp40(AS) primers
(1.0 kb)
pBluescriptSK+
(Stratagene)
SmaI
PstI
EcoRI
EcoRV
HindIII
ClaI
SalI
XhoI
ApaI
BamHI
SpeI
XhaI
NotI
hp35
pSK-hp35
(3.8 kb)
SmaI
(3.0 kb)
SmaI
(3.0 kb)
NcoI
hp40
NotI
pSK-hp40
(4.0 kb)
EcoRV
(3.0 kb)
NcoI
EcoRI
PstI
SmaI
BamI
SpeI
XbaI
NotI
EcoRV
HindIII
ClaI
SalI
XhoI
ApaI
IRES
pSK-IRES
(3.5 kb)
RT-PCR with IRES(S)
& IRES(AS) primers
EcoRV
(0.5 kb)
(0.5 kb)
EcoRV
NotI
T4
(0.8 kb)
EcoRV
(3.5 kb)
NcoI
NotI
(1.0 kb)
SmaI
PstI
EcoRI
EcoRV
HindIII
ClaI
SalI
XhoI
ApaI
KpnI
BamHI
SpeI
XbaI
NcoI
EcoRI
PstI
SmaI
BamI
SpeI
XbaI
NotI
hp35
IRES
pSK-hp35IRES
(4.3 kb)
SmaI
ClaI
Re-ligation
NcoI
NotI
(4.3 kb)
BamHI
SpeI
XbaI
NcoI
SalI
XhoI
ApaI
kpnI
hp35
hp40
NotI
IRES
pSK-hp35/IRES/hp40
(5.3 kb)

FIG. 17B

1st PCR
primer: T7(S) & hp40-N222L(AS)
template: pSK-hp40

2nd PCR
primer: hp40-N222L(S) & T3(AS)
template: pSK-hp40

3rd PCR
primer: hp40-N222L(S) & T3(AS)
template: product mixture of 1st PCR & 2nd PCR

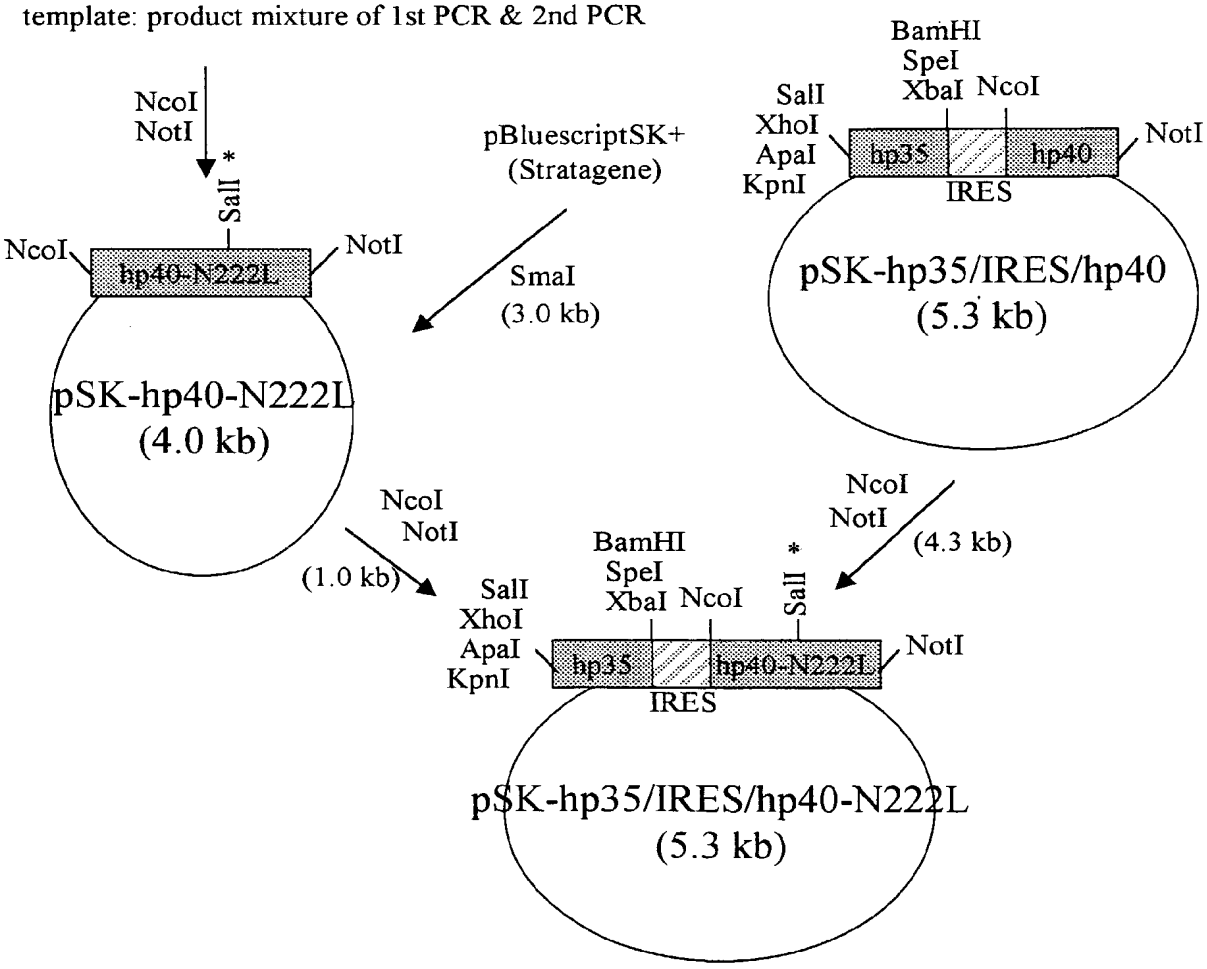


FIG. 17C

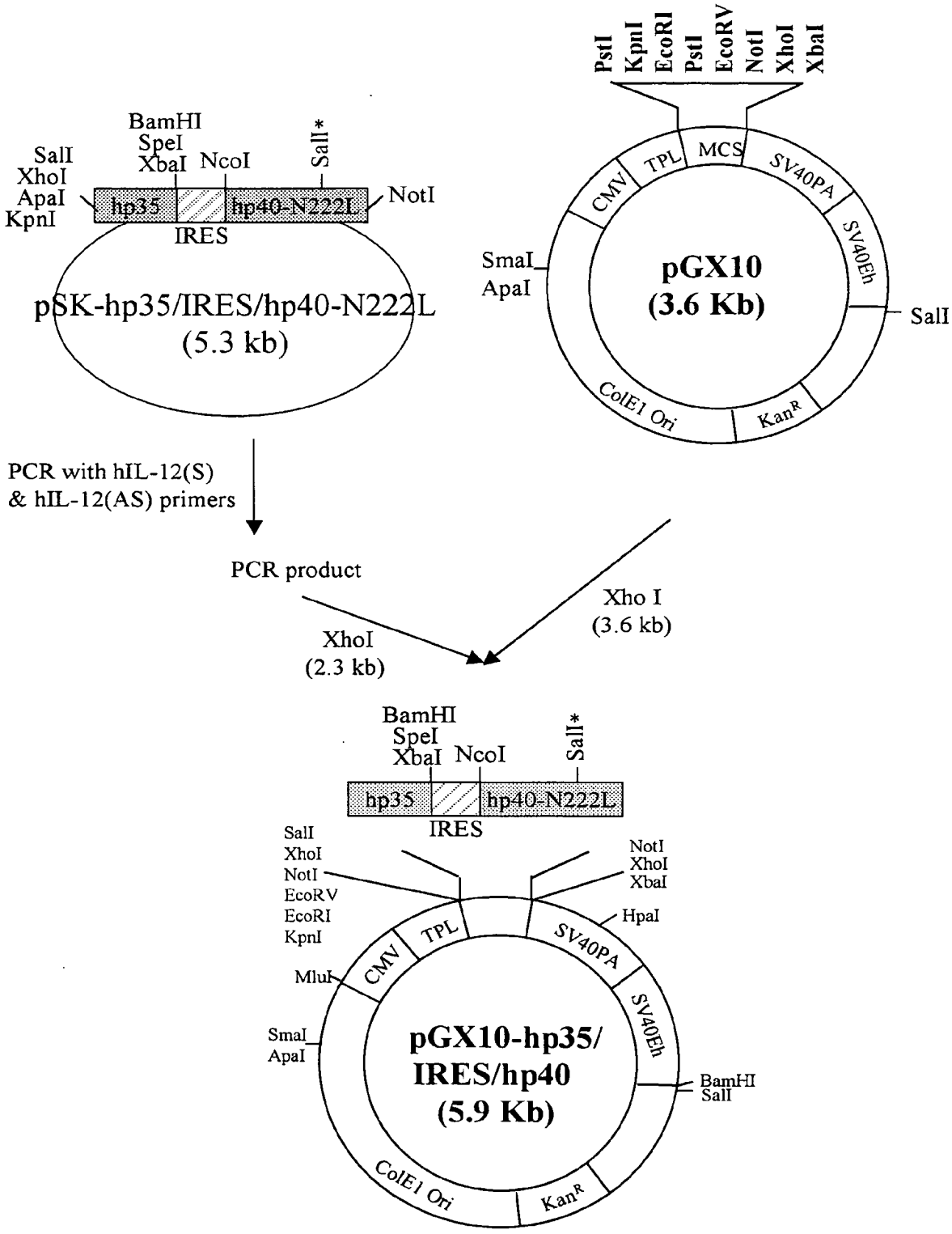

PstI KpnI EcoRI PstI EcoRV NotI XhoI XbaI
MCS
TPL
CMV
SV40PA
SV40Eh
pGX10
(3.6 Kb)
SmaI
ApaI
SalI
ColE1 Ori
Kan^R
BamHI
SpeI
XbaI
NcoI
SalI*
SalI
XhoI
ApaI
KpnI
hp35
hp40-N222L
NotI
IRES
pSK-hp35/IRES/hp40-N222L
(5.3 kb)
PCR with hIL-12(S)
& hIL-12(AS) primers
PCR product
XhoI
(2.3 kb)
Xho I
(3.6 kb)
BamHI
SpeI
XbaI
NcoI
SalI*
hp35
hp40-N222L
IRES
SalI
XhoI
NotI
EcoRV
EcoRI
KpnI
NotI
XhoI
XbaI
HpaI
MluI
CMV
TPL
SV40PA
SV40Eh
SmaI
ApaI
pGX10-hp35/
IRES/hp40
(5.9 Kb)
BamHI
SalI
ColE1 Ori
Kan^R

FIG. 18

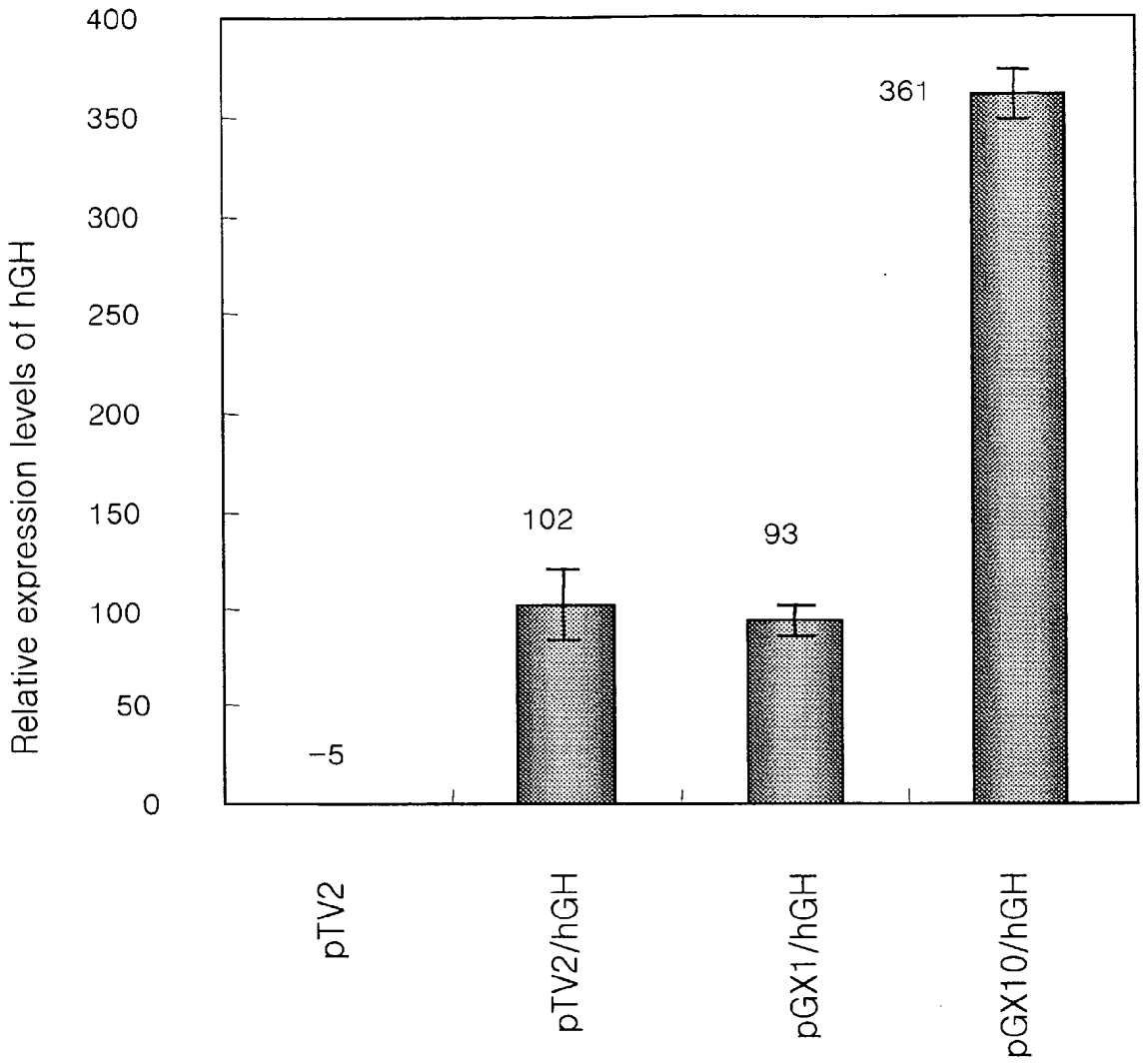

Relative expression levels of hGH
400
350
300
250
200
150
100
50
0
-5
102
93
361
pTV2
pTV2/hGH
pGX1/hGH
pGX10/hGH

FIG. 19

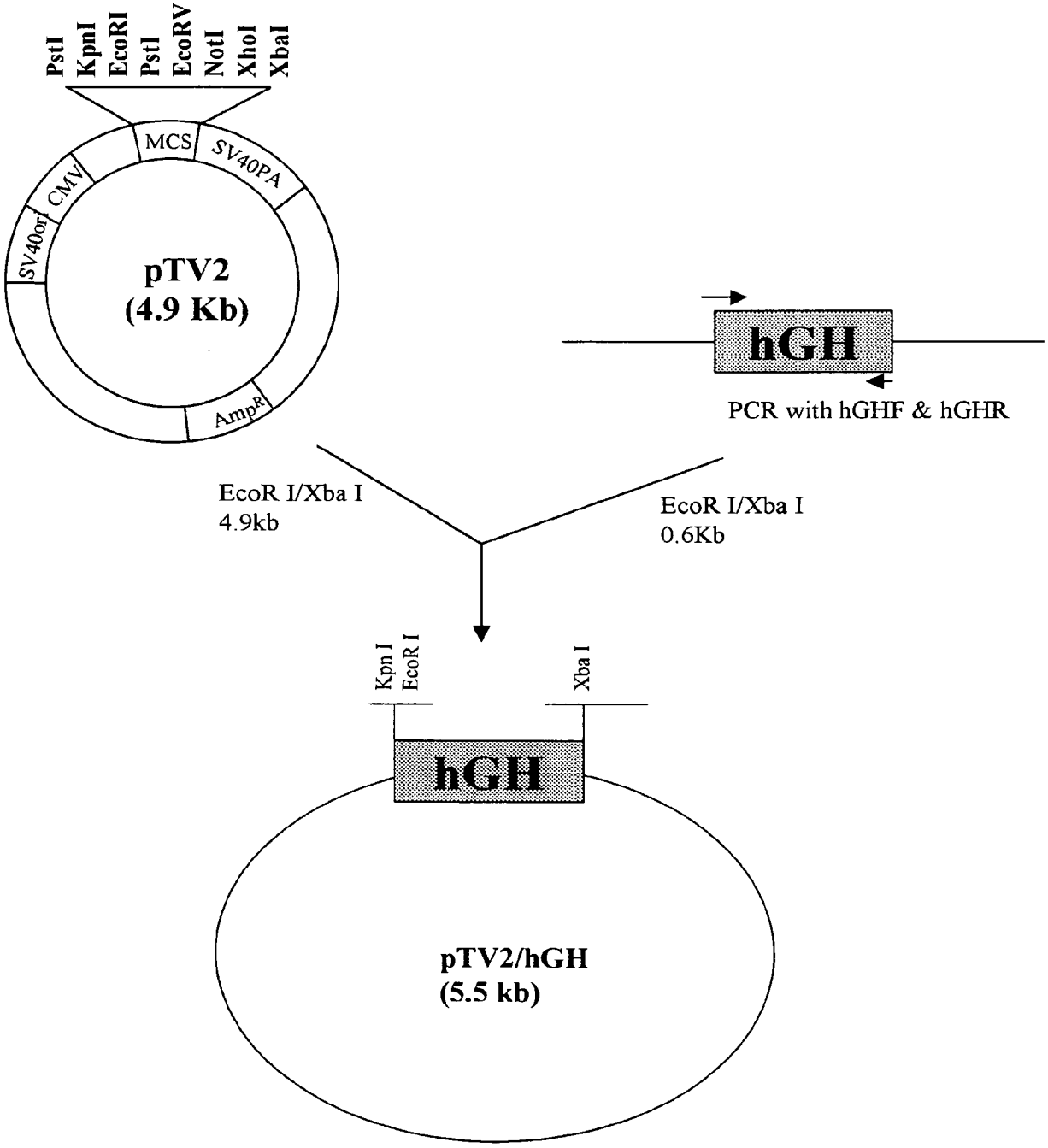

PstI
KpnI
EcoRI
PstI
EcoRV
NotI
XhoI
XbaI
MCS
SV40PA
CMV
SV40or
pTV2
(4.9 Kb)
AmpR
hGH
PCR with hGHF & hGHR
EcoR I/Xba I
4.9kb
EcoR I/Xba I
0.6Kb
Kpn I
EcoR I
Xba I
hGH
pTV2/hGH
(5.5 kb)

FIG. 20

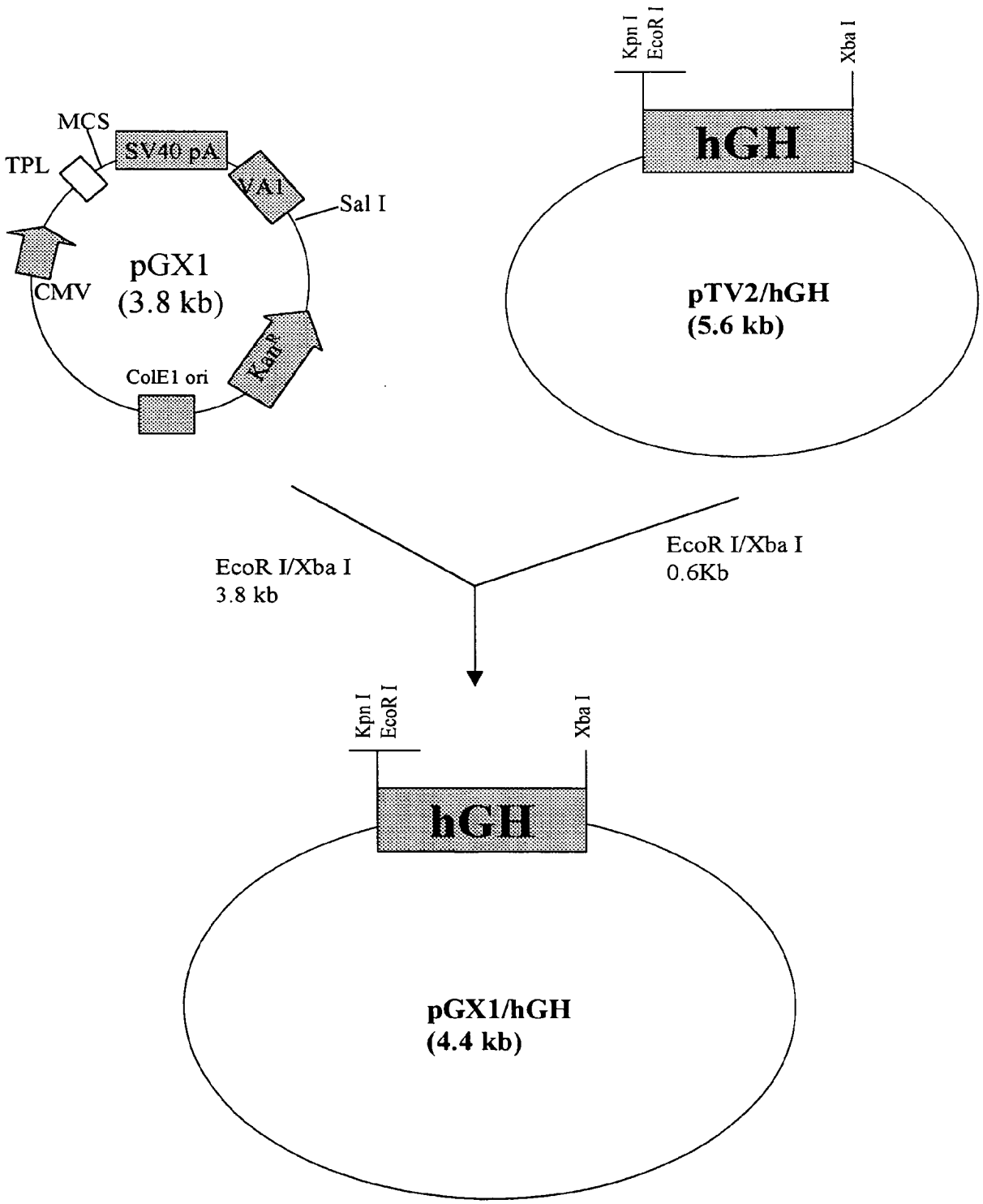

MCS
SV40 pA
TPL
VA1
Sal I
pGX1
(3.8 kb)
CMV
ColE1 ori
Kan
Kpn I
EcoR I
Xba I
hGH
pTV2/hGH
(5.6 kb)
EcoR I/Xba I
3.8 kb
EcoR I/Xba I
0.6Kb
Kpn I
EcoR I
Xba I
hGH
pGX1/hGH
(4.4 kb)

FIG. 21

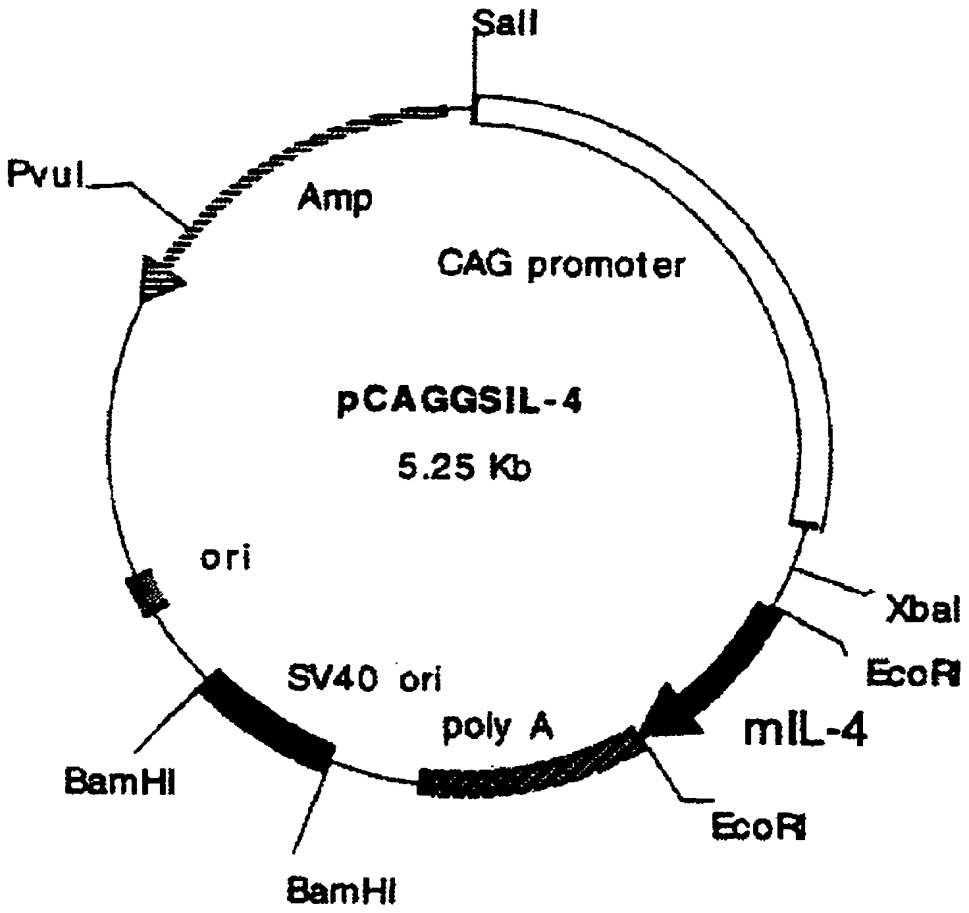

SalI
PvuI
Amp
CAG promoter
pCAGGSIL-4
5.25 Kb
ori
XbaI
EcoRI
SV40 ori
poly A
mIL-4
BamHI
EcoRI
BamHI

FIG. 22

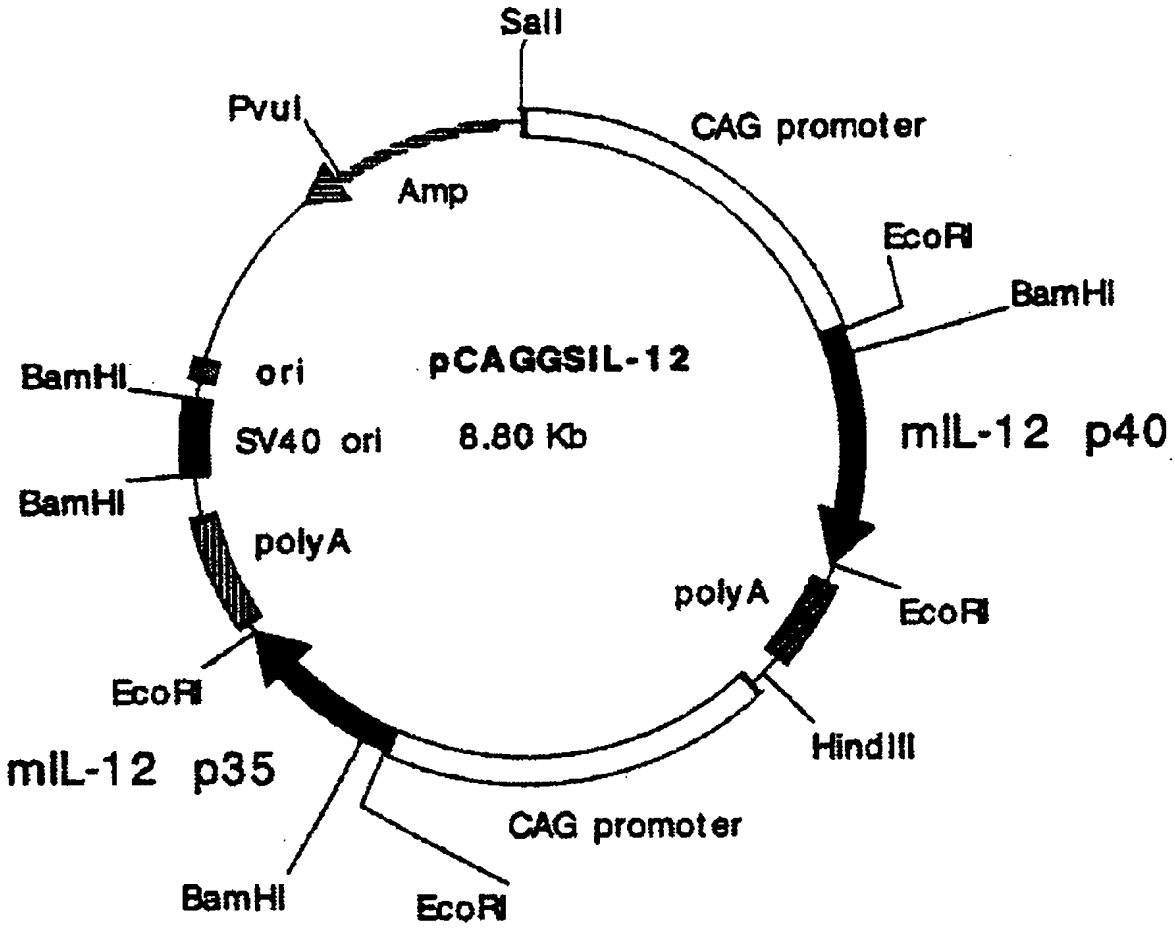

SalI
PvuI
CAG promoter
Amp
EcoRI
BamHI
BamHI
ori
pCAGGSIL-12
mIL-12 p40
SV40 ori
8.80 Kb
BamHI
polyA
polyA
EcoRI
EcoRI
HindIII
mIL-12 p35
CAG promoter
BamHI
EcoRI

FIG. 23

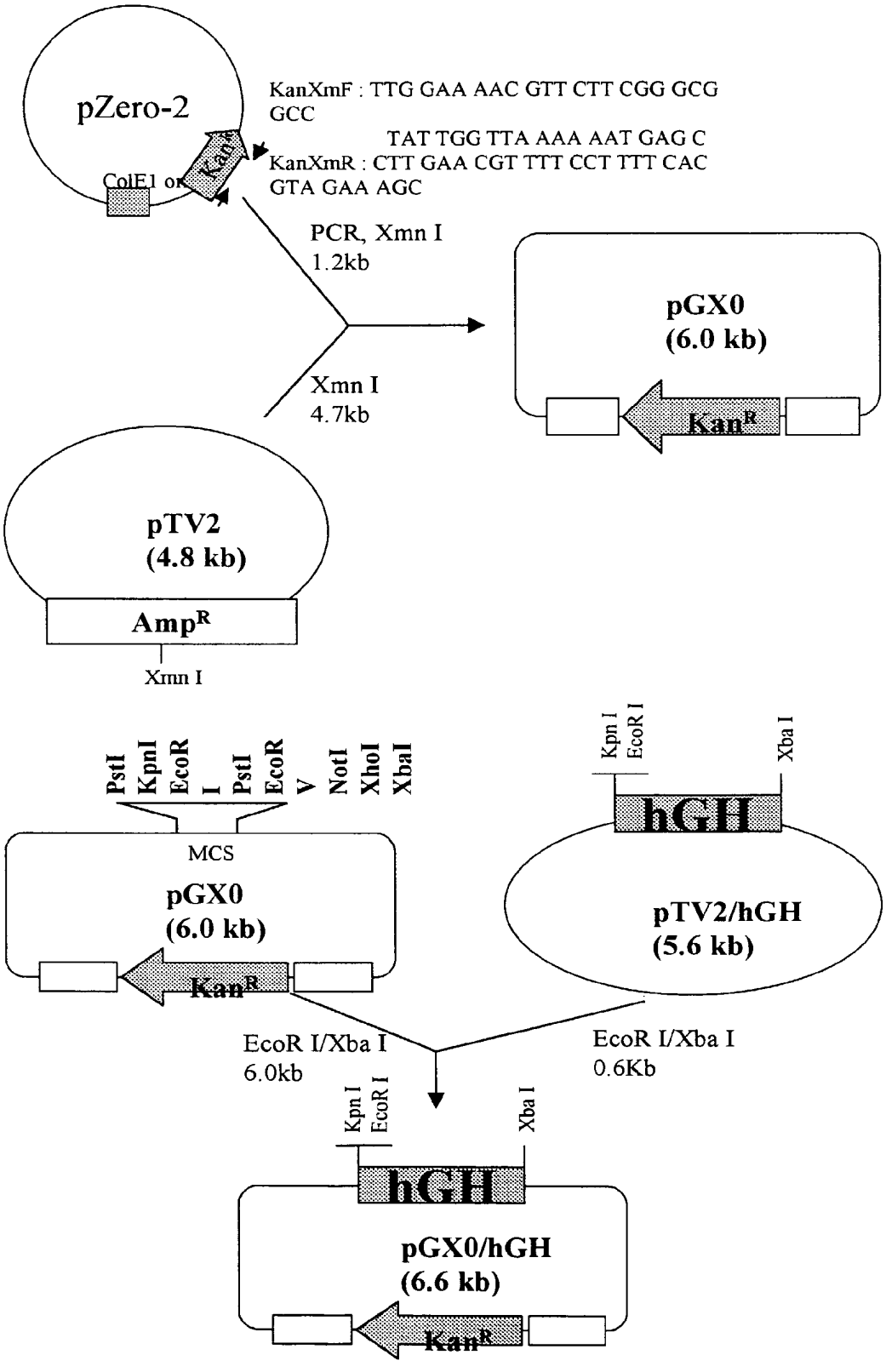

pZero-2
ColE1 ori
Kan^R
KanXmF : TTG GAA AAC GTT CTT CGG GCG GCC
TAT TGG TTA AAA AAT GAG C
KanXmR : CTT GAA CGT TTT CCT TTT CAC GTA GAA AGC
PCR, Xmn I
1.2kb
Xmn I
4.7kb
pGX0
(6.0 kb)
Kan^R
pTV2
(4.8 kb)
Amp^R
Xmn I
PstI
KpnI
EcoR I
PstI
EcoR V
NotI
XhoI
XbaI
MCS
pGX0
(6.0 kb)
Kan^R
Kpn I
EcoR I
Xba I
hGH
pTV2/hGH
(5.6 kb)
EcoR I/Xba I
6.0kb
EcoR I/Xba I
0.6Kb
Kpn I
EcoR I
Xba I
hGH
pGX0/hGH
(6.6 kb)
Kan^R

FIG. 24

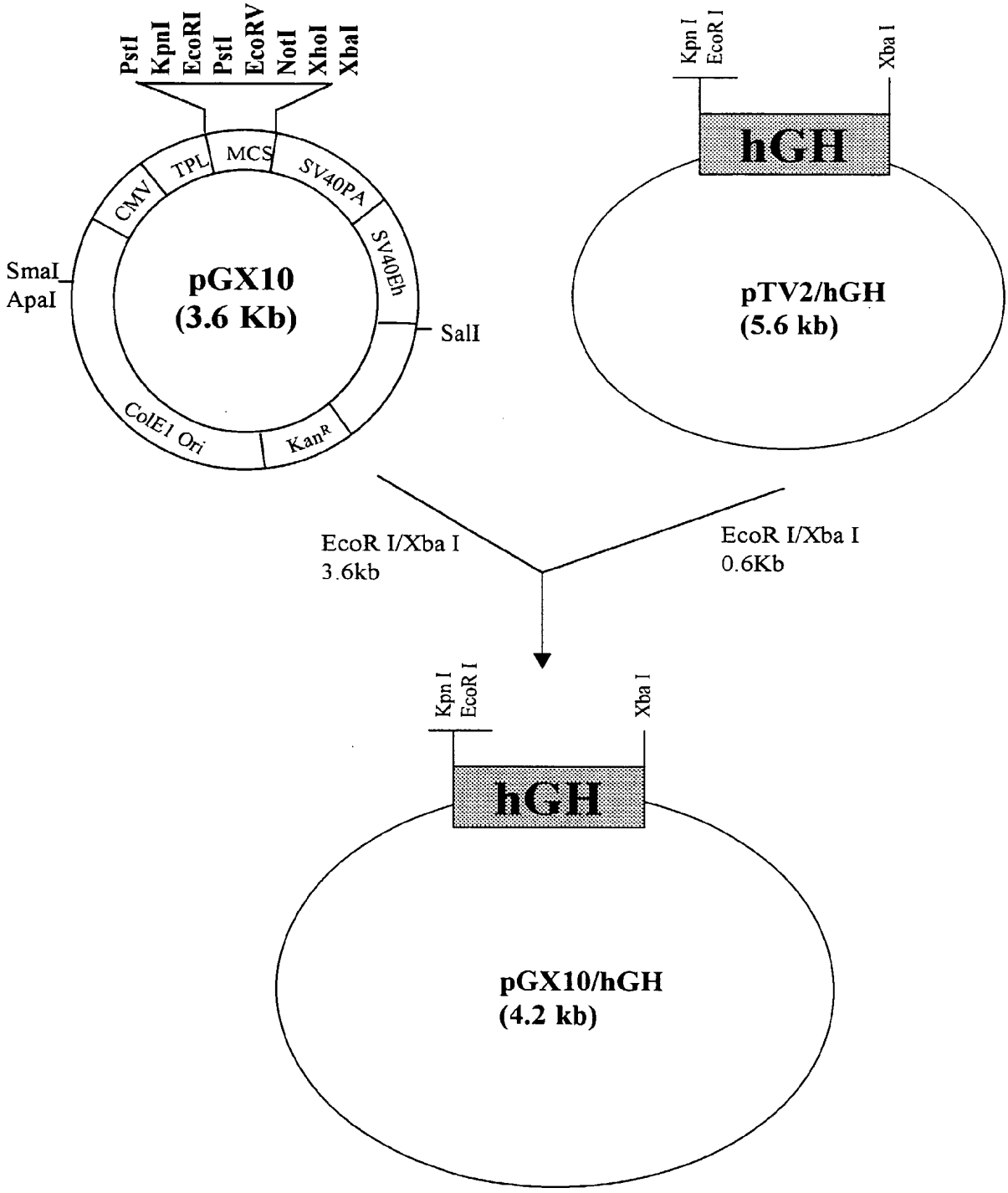

PstI
KpnI
EcoRI
PstI
EcoRV
NotI
XhoI
XbaI
CMV
TPL
MCS
SV40PA
SV40Eh
SmaI
ApaI
pGX10
(3.6 Kb)
SalI
ColE1 Ori
KanR
Kpn I
EcoR I
Xba I
hGH
pTV2/hGH
(5.6 kb)
EcoR I/Xba I
3.6kb
EcoR I/Xba I
0.6Kb
Kpn I
EcoR I
Xba I
hGH
pGX10/hGH
(4.2 kb)

FIG. 25

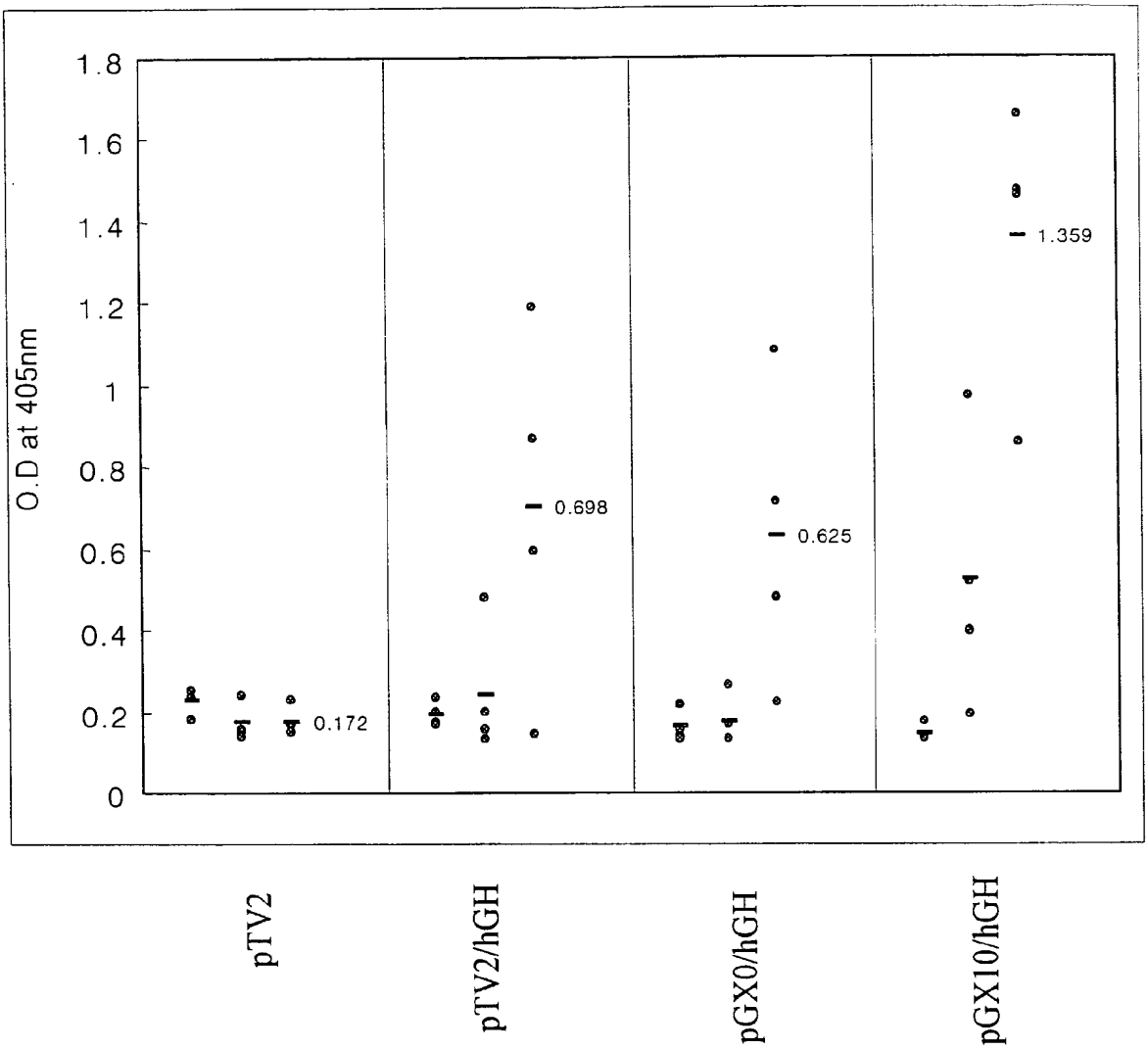
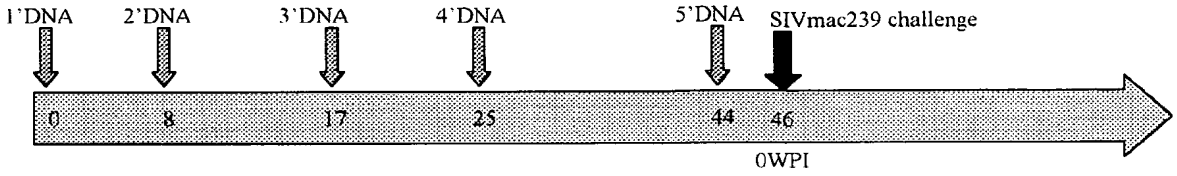

O.D at 405nm
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0.172
0.698
0.625
1.359
pTV2
pTV2/hGH
pGX0/hGH
pGX10/hGH

FIG. 26



Group (monkey#)	Immunogen (weeks post the 1st immunization) 1'DNA (0week)	2'DNA (8 weeks)	3'DNA or protein (17weeks)	4'DNA or protein (25 weeks)	5'DNA or protein (44 weeks)	Challenge (46 weeks)
1 (8645, 8772, 8774)	pTV2 : 800ug	◀	◀	◀	◀	SIVmac239 dose: 10 MID_{50} route: i.v
2+5 (9994, 9995, 10001)	pTV2-SIV/GE : 400ug pTV2-SIV/dpol : 400ug	◀	◀	◀	◀	
3 (10003, 10005, 10011)	pGX10-SIV/GE : 400ug pGX10-SIV/dpol : 400ug pGX10-SIV/VN : 400 ug pGX10-SIV/TV : 400 ug	◀	◀	◀	◀	
4 (10012, 10013, 10014)	pGX10-SIV/GE : 400ug pGX10-SIV/dpol : 400ug pGX10-SIV/VN : 400 ug pGX10-SIV/TV : 400 ug pGX10-hIL-12m : 400 ug	◀	◀	◀	◀	

Schematic of Immunization schedule for each group of monkeys. Monkey designation (four or five digit number), immunogens, immunization intervals, and challenge time were shown.

FIG. 27

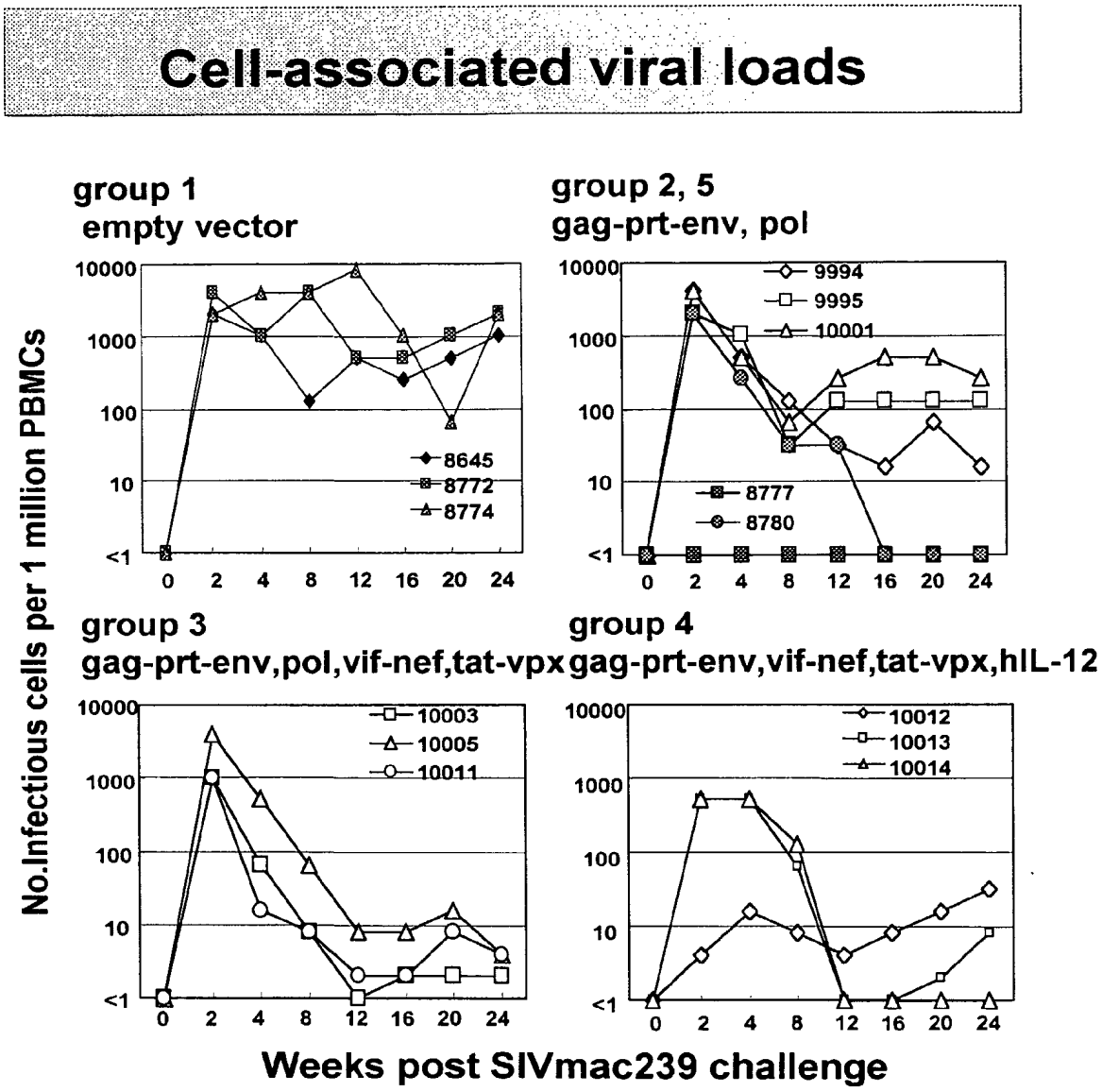

Cell-associated viral loads
group 1
empty vector
group 2, 5
gag-prt-env, pol
group 3
gag-prt-env,pol,vif-nef,tat-vpx
group 4
gag-prt-env,vif-nef,tat-vpx,hIL-12
No.Infectious cells per 1 million PBMCs
10000
1000
100
10
<1
0 2 4 8 12 16 20 24
8645
8772
8774
9994
9995
10001
8777
8780
10003
10005
10011
10012
10013
10014
Weeks post SIVmac239 challenge

FIG. 28

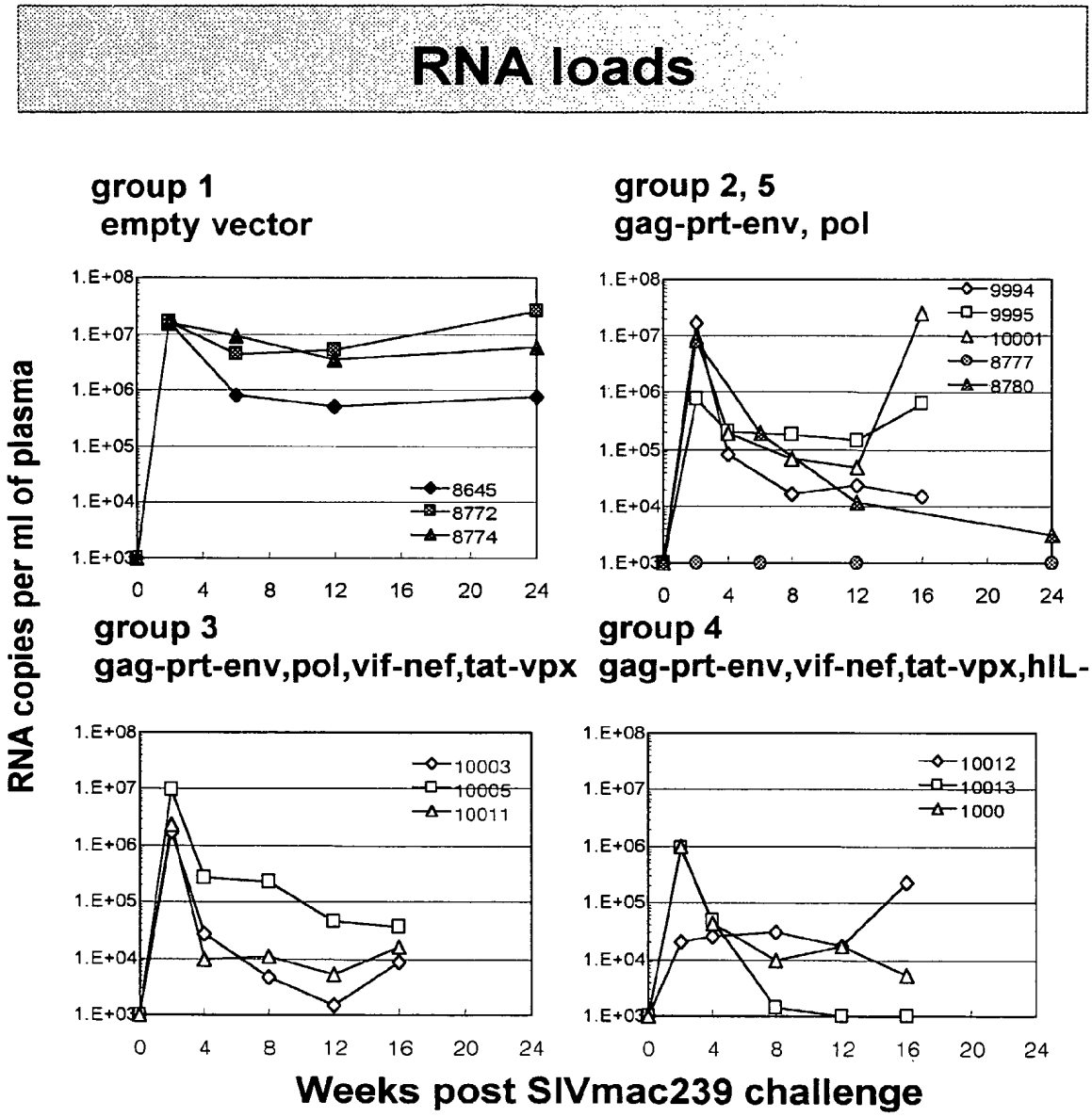

RNA loads
group 1
empty vector
group 2, 5
gag-prt-env, pol
group 3
gag-prt-env,pol,vif-nef,tat-vpx
group 4
gag-prt-env,vif-nef,tat-vpx,hIL-12
RNA copies per ml of plasma
1.E+08
1.E+07
1.E+06
1.E+05
1.E+04
1.E+03
0
4
8
12
16
20
24
8645
8772
8774
9994
9995
10001
8777
8780
10003
10005
10011
10012
10013
1000
Weeks post SIVmac239 challenge

FIG. 29

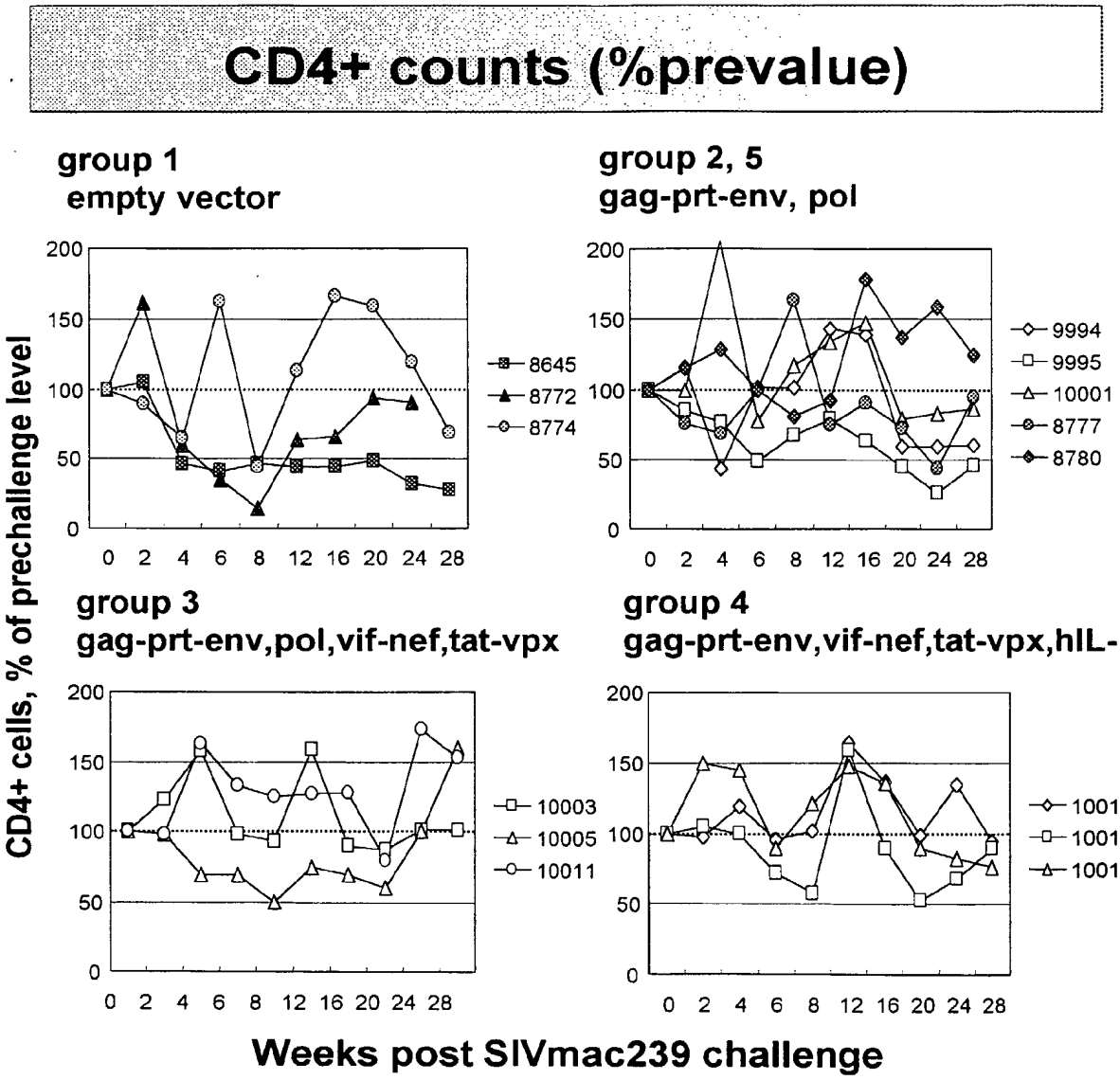

CD4+ counts (%prevalue)
group 1
empty vector
group 2, 5
gag-prt-env, pol
group 3
gag-prt-env,pol,vif-nef,tat-vpx
group 4
gag-prt-env,vif-nef,tat-vpx,hIL-1
CD4+ cells, % of prechallenge level
200
150
100
50
0
0 2 4 6 8 12 16 20 24 28
8645
8772
8774
9994
9995
10001
8777
8780
10003
10005
10011
10012
10013
10014
Weeks post SIVmac239 challenge

FIG. 30

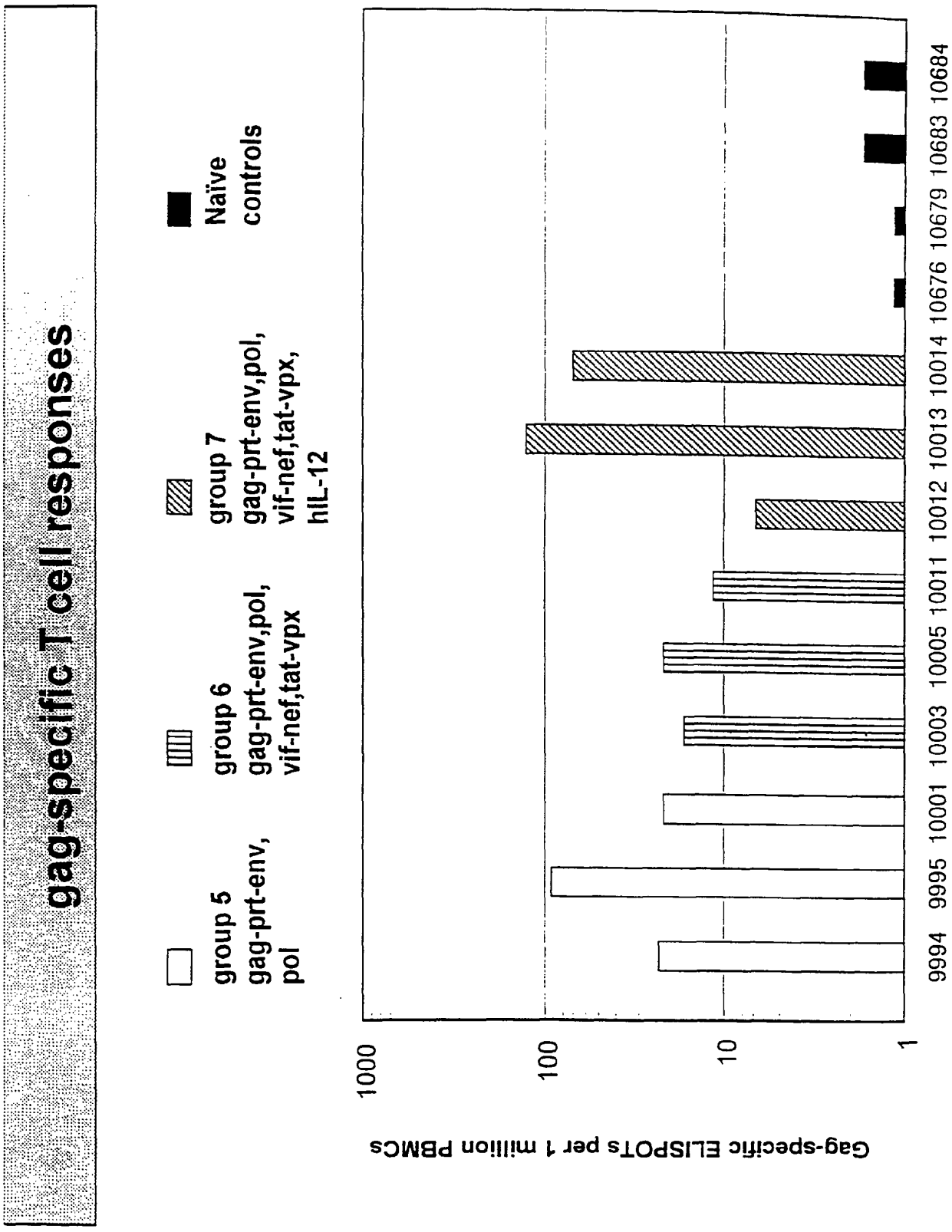

gag-specific T cell responses
group 5
gag-prt-env, pol
group 6
gag-prt-env,pol, vif-nef,tat-vpx
group 7
gag-prt-env,pol, vif-nef,tat-vpx, hIL-12
Naïve controls
Gag-specific ELISPOTs per 1 million PBMCs
1000
100
10
1
9994
9995
10001
10003
10005
10011
10012
10013
10014
10676
10679
10683
10684

FIG. 31

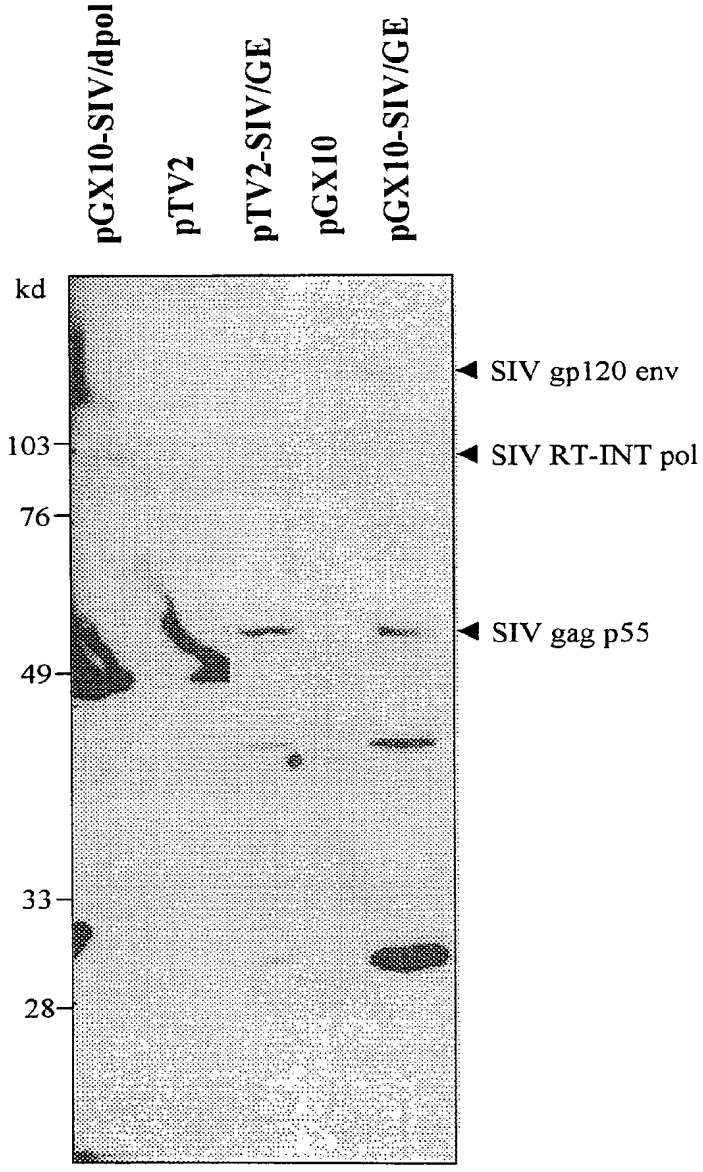

pGX10-SIV/dpol
pTV2
pTV2-SIV/GE
pGX10
pGX10-SIV/GE
kd
103
76
49
33
28
SIV gp120 env
SIV RT-INT pol
SIV gag p55

FIG. 32

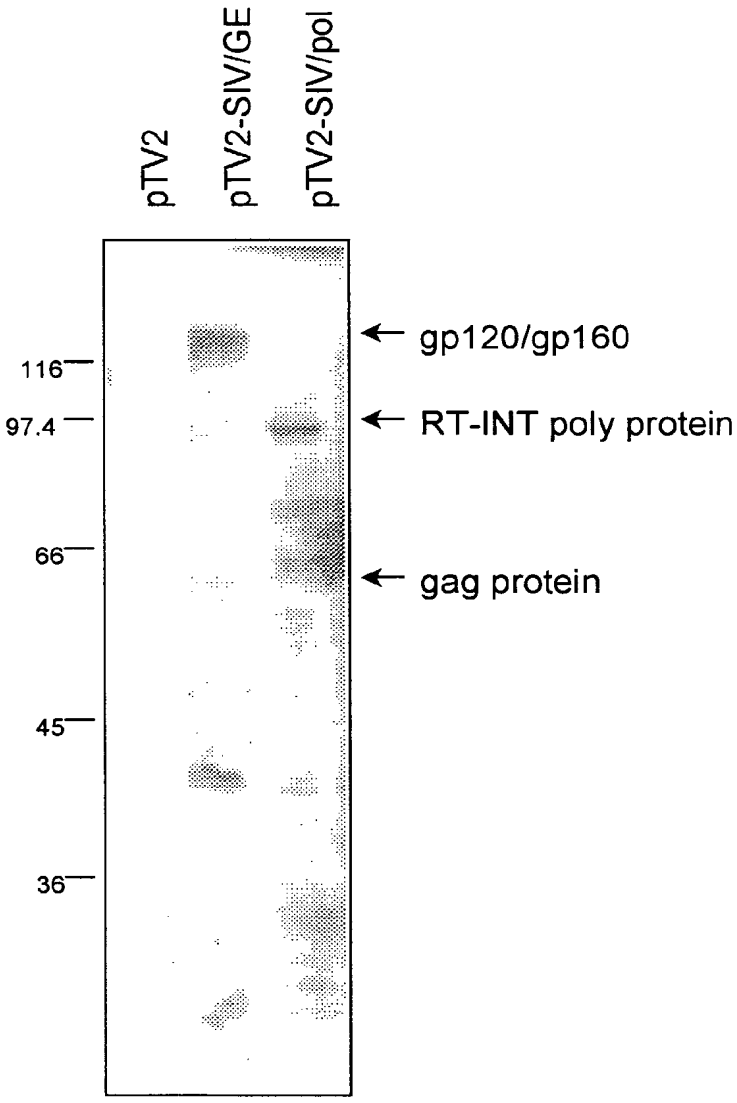

pTV2
pTV2-SIV/GE
pTV2-SIV/pol
116
97.4
66
45
36
gp120/gp160
RT-INT poly protein
gag protein

FIG. 33

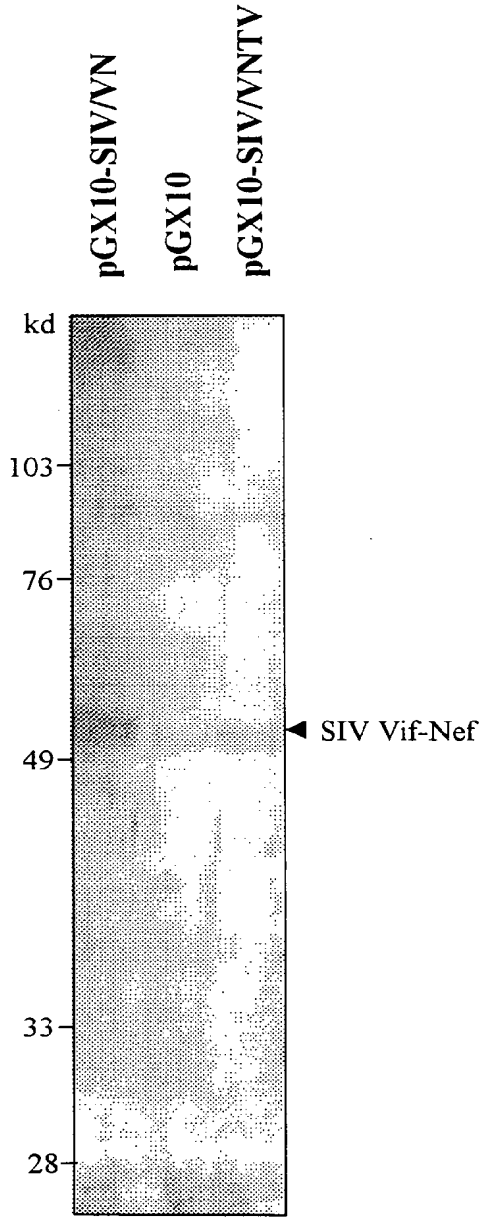
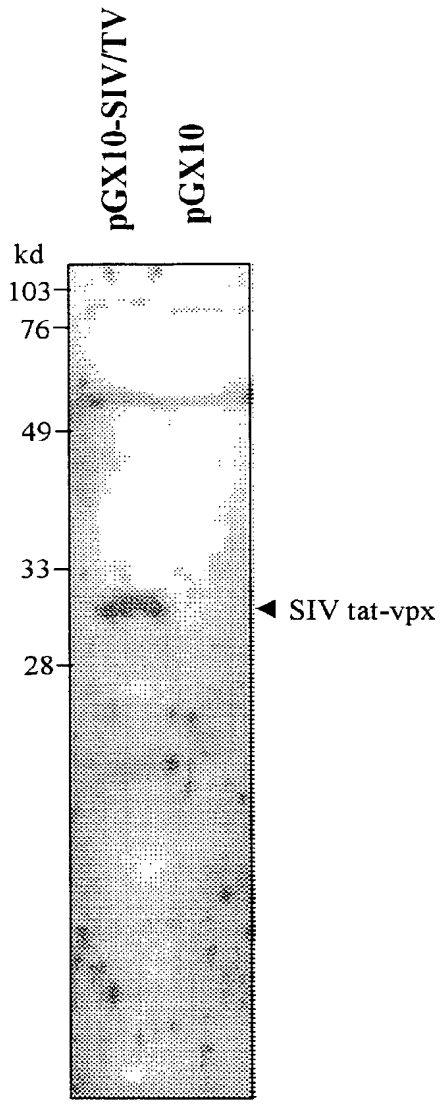


FIG. 34



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<170> KopatentIn 1.71

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Sequence Listing

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<220>
<223> primer

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aatattgggc ccgaacatgt gagcaaaagg ccag 34

<210> 5
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 5
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<210> 6
<211> 22
<212> DNA
<213> Artificial Sequence

Sequence Listing

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<211> 20
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<213> Artificial Sequence

<220>
<223> primer

<400> 7
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<210> 8
<211> 32
<212> DNA
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<220>
<223> primer

<400> 8
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<211> 20
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<220>

Sequence Listing

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<220>
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<210> 12
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<212> DNA
<213> Artificial Sequence

<220>
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Sequence Listing

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<213> Artificial Sequence

<220>
<223> primer

<400> 13
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<210> 14
<211> 23
<212> DNA
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<220>
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<400> 14
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<220>
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Sequence Listing

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<220>
<223> primer

<400> 17
tatggcgcgc ctggaggagg aaaagagg 28

<210> 18
<211> 38
<212> DNA
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<220>
<223> primer

Sequence Listing

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<223> primer

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<210> 20
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<212> DNA
<213> Artificial Sequence

<220>
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<210> 21
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<400> 21

Sequence Listing

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<210> 22
<211> 26
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<210> 23
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<213> Artificial Sequence

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<400> 23
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<210> 24
<211> 28
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<400> 24
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Sequence Listing

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aaagcggccg caatctagag tttgatgcag aagatgta 38

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<220>
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<400> 26
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<210> 27
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<213> Artificial Sequence

<220>
<223> primer

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<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 29
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<210> 30
<211> 23
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<220>
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<400> 30
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Sequence Listing

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<220>
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<220>
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<210> 33
<211> 24
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<223> primer

<400> 33
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<210> 34

Sequence Listing

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<220>
<223> primer

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<220>
<223> primer

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<210> 36
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<223> primer

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Sequence Listing

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<210> 40
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Sequence Listing

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ccctctagac tttggtagag aaacttg 27

<210> 42
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<210> 43
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Sequence Listing

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cgagcggccg cctacagatc attaatgtc 29

<210> 44
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cccgggaaag tcctgccgcg cctcg 25

<210> 45
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<400> 45
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<210> 46
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agagcaccat gggtcaccag cagttgg 27

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<400> 47
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Sequence Listing

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ttgccatggc catatttatc a 21

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<400> 50
gtaatacgac tcactatagg gc 22

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Sequence Listing

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Sequence Listing

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<400> 59
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<220>
<223> vecter

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ccgcccaacg acccccgccc attgacgtca ataatgacgt atgttcccat agtaacgcca 180

atagggactt tccattgacg tcaatgggtg gagtatttac ggtaaactgc ccacttggca 240

gtacatcaag tgtatcatat gccaagtacg ccccctattg acgtcaatga cggtaaatgg 300

cccgcctggc attatgccca gtacatgacc ttatgggact ttcctacttg gcagtacatc 360

tacgtattag tcatcgctat taccatggtg atgcggtttt ggcagtacat caatgggcgt 420

Sequence Listing

ggatagcggt ttgactcacg gggatttcca agtcttcacc ccattgacgt caatgggagt 480

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acgcaaatgg gcggtaggcg tgtacggtgg gaggtctata taagcagagc tctctggcta 600

actagagaac ccactgctta ctggcttatc gaaattaata cgactcacta tagggagacc 660

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gaggacaaac tcttcgcggt ctttccagta ctcttggatc ggaaacccgt cggcctccga 780

acggtactcc gccaccgagg gacctgagcg agtccgcatc gaccggatcg gaaaacctct 840

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ctgggcggag ttaggggcgg gatgggcgga gttaggggcg ggactatggt tgctgactaa 1560

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Sequence Listing

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ggaagcggtc agcccattcg ccgccaagct cttcagcaat atcacgggta gccaacgcta 1860

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cgaatagcct ctccacccaa gcggccggag aacctgcgtg caatccatct tgttcaatca 2520

tgcgaaaacga tcctcatcct gtctcttgat cagatcttga tcccctgcgc catcagatcc 2580

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ccccagctgg caattccggt tcgcttgctg tccataaaac cgcccagtct agctatcgcc 2700

atgtaagccc actgcaagct acctgctttc tctttgcgct tgcgttttcc cttgtccaga 2760

tagcccagta gctgacattc atccggggtc agcaccgttt ctgcggactg gctttctacg 2820

Sequence Listing

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agttttcgtt ccactgagcg tcagaccccg tagaaaagat caaaggatct tcttgagatc 2940

ctttttttct gcgcgtaatc tgctgcttgc aaacaaaaaa accaccgcta ccagcggtgg 3000

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gatttttgtg atgctcgtca ggggggcgga gcctatggaa aaacgccagc aacgcggcct 3540

ttttacggtt cctgggcttt tgctggcctt ttgctcacat gttcgggccc aatcgacccg 3600

ggcgacggcc agtgaattgt accgatgtac gggccagata t 3641

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR02/00855

A. CLASSIFICATION OF SUBJECT MATTER

IPC7 C12N 15/63, A61K 39/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N 15/63, A61K 39/12

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korea Patents and Applications for Invention since 1975

Electronic data base consulted during the intertnational search (name of data base and, where practicable, search terms used)
PubMed, CA, Delphion, "DNA vaccine", "AIDS", "vector"

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Lee, A.H. et al., Vaccine, 17, 473-79, 1999.	1-102
A	Harms, J.S. et al., Braz. J. Med. Biol. Res., 32(2), 155-62, 1999.	1-102
A	Lee, S.W. et al., J. Virol., 72, 8430-36, 1998.	1-102
A	KR 2001-0054338 A (Geneccin Co. Ltd.) Jul 2, 2001.	1-102
A	US 5,698,432 A (Retroscreen Ltd.), Dec 16, 1997.	1-102
A	US 6,004,799 A (The Regents of the Univ. of Califonia), Dec 21, 1999.	1-102

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:
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